REMARKS

Status of the Claims

In the April 18 Office Action, the Examiner acknowledged that Claims 44, 51-53 and 56-63 had been cancelled, and that Claims 64 and 65 had been added, but then stated that Claims 1-43, 45-50 and 54-63 are pending. Instead, it is noted that Claims 1-43, 45-50, 54-55, and 64-65 were currently pending and are under examination (new claims are also now added). It is believed that this was a clerical error in the statement of the status of the claims and in the statements of the claims under rejection under 35 U.S.C. § 102(f) and 35 U.S.C. § 112, first paragraph, since the Examiner explicitly refers to Claims 64 and 65 in the rejections of the claims under 35 U.S.C. § 112, first paragraph, and does not explicitly refer to Claims 56-63 in any of the rejections, indicating that the actually pending claims were in fact examined. The rejections are addressed below according to this understanding.

Amendments to the Claims

Claims 45-48, 54, 64, and 65 have been amended to be limited to the apoptosis-inducing protein being Fas ligand and the apoptosis-inhibiting protein being CrmA. Support for this amendment is found in original Claim 10 (CrmA) and 11 (Fas ligand), page 20, lines 12-20; page 22, lines 12-13; and in the Examples. Support for the amendment to administration of the vector or cell "at or adjacent to the site of the cancer cells" is supported in original Claim 54, page 55, lines 11-14, or in the Examples.

New Claims 66 and 67 are supported by prior Claim 55 and page 55, lines 25-29.

New Claims 68-70 are supported by original Claim 17, page 33, line 11, and the Examples.

Rejection of Claims 1-43 Under 35 U.S.C. § 102(a):

The Examiner has rejected Claims 1-43 under 35 U.S.C. § 102(a), contending that these claims are anticipated by Shinoura et al., *Human Gene Therapy* 9(18):2683-2689, December 10, 1998. The Examiner asserts that Shinoura et al. teaches a method to propagate a recombinant adenovirus vector comprising a nucleic acid molecule encoding Fas ligand, where the method includes culturing cells transfected with the CrmA gene and a recombinant adenoviral vector

encoding Fas ligand.

Applicants traverse the rejection of Claims 1-43 under 35 U.S.C. § 102(a). Specifically, it is submitted that Shinoura et al. is not an effective reference against the present claims, because the claimed subject matter in Claims 1-43 was invented by the present inventors prior to the effective date of Shinoura et al., which is the publication date, December 10, 1998. Enclosed herewith is a Declaration under 37 CFR § 1.131 executed by all of the present inventors. This Declaration provides evidence of conception and actual reduction to practice of the invention as claimed in Claims 1-43 at a date prior to December 10, 1998. As required by 37 CFR § 1.131, the Declaration affirms that the acts relied upon to establish actual reduction to practice were carried out in the United States.

Therefore, the Examiner is respectfully requested to withdraw the rejection of Claims 1-43 under 35 U.S.C. § 102(a).

Rejection of Claims 1-43, 45-50, 54-55[sic] and 64-65[sic] Under 35 U.S.C. § 102(f):

The Examiner has rejected Claims 1-43, 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 102(f), contending that these claims are anticipated by Hedlund et al., *Cell Death and Differentiation* 6:155-182, February 1999. Referring to the discussion of the status of the claims above, it is believed that the Examiner intended to apply the rejection to these pending claims, and not to the cancelled claims.

Applicants traverse the rejection of Claims 1-43, 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 102(f), because the publication of Hedlund et al. is not effective prior art against the present claims. Specifically, Hedlund et al. discloses subject matter invented by only by coinventors Richard C. Duke or Jerome B. Schaack, rather than subject matter derived from the authors of Hedlund et al., notwithstanding the authorship of the article. Enclosed herewith is a Declaration under 37 CFR 1.132 executed by inventors Richard C. Duke or Jerome B. Schaack, averring that the other authors of Hedlund et al. were merely working at the direction of Richard C. Duke or Jerome B. Schaack, and are not inventors of the subject matter claimed in the present application.

Therefore, the Examiner is respectfully requested to withdraw the rejection of Claims 1-43, 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 102(f).

Objection to the Specification and Rejection of Claims 45-50, 54-55[sic] and 64-65[sic] Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 112, first paragraph, on the basis of enablement. Specifically, the Examiner contends that at best, the specification teaches construction of a recombinant adenovirus encoding Fas ligand and that Fas ligand suppresses T lymphocyte-mediated rejection of transplanted islet cells in diabetic rats. The Examiner asserts that the specification fails to disclose a method of inducing apoptosis in cancer cells of a recipient mammal by introducing into the mammal a recombinant viral vector that encodes a protein that induces apoptosis, by introducing a recombinant viral vector that encodes a protein that inhibits apoptosis and a protein that induces apoptosis, or by introducing a viral vector comprising at least a portion of SEQ ID NO:4 and a nucleic acid molecule encoding Fas ligand. The Examiner refers to several different references in support of the position that gene therapy is unpredictable. The Examiner also contends that systemic administration of the viral vector would lead to expression of Fas ligand in any tissue or cell that would be deleterious to the subject. The Examiner asserts that one of the greatest challenges facing gene therapy is the efficient transfer and stable expression of transgenes by appropriate tissues. The Examiner further states that it is unclear how one would target tumor metastases as they spread to a different part of the body. Finally, the Examiner considers that administration by any route is unpredictable without specific guidance.

The rejection of Claims 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 112, first paragraph, is respectfully traversed. Initially, to expedite prosecution, Claims 45-50, 54-55 and 64-65, as well as the new method Claims 66-70, have been limited to the use of Fas ligand as the apoptosis-inducing protein, where applicable, and to the use of CrmA as the apoptosis-inhibiting protein, where applicable. In addition, these claims have been amended to recite that the viral vector is introduced at or adjacent to the site of the cancer cells in the mammal. Support for these amendments is discussed above.

With respect to the allegation that the specification does not provide any support for a method of inducing apoptosis in cancer cells of a recipient mammal by introducing into the mammal a recombinant viral vector that encodes Fas ligand, this is not correct. Applicants respectfully refer the Examiner to the present specification and working Examples 11-13,

Figures 3 and 4 and Tables 2 and 3. These examples demonstrate: (1) that a recombinant viral vector encoding Fas ligand can be efficiently transduced into and expressed by mammalian cells, including tumor cells, both *in vitro* and *in vivo*; (2) that both mouse and human Fas ligand expressed by a recombinant viral vector induced apoptosis in a variety of different cells *in vitro*, showing significantly better efficacy than anti-Fas antibodies; (3) expression of recombinant Fas ligand in tumor cells *in vivo* inhibited tumor growth and tumor size, including in tumor cells that were resistant to Fas ligand *in vitro*; and (4) injection of recombinant viral vector encoding Fas ligand into existing tumor cells *in vivo* induced apoptosis at the site of the tumor. As discussed in Example 13, the inventors have also discovered that in addition to the direct anti-tumor effects of Fas ligand on tumors, the effects of Fas ligand appeared to also be due in part to an apoptotic effect on other cells at the site of injection, such as neutrophils, which then potentiated an inflammatory anti-tumor response and reduced tumor growth/size (this "bystander" effect has been subsequently further validated by multiple investigators, *e.g.,* see Hyer et al., *Cancer Gene Ther.* 10:330-339, 2003). As discussed in more detail below, it is now also known that the introduction of Fas ligand to a cancer has an apoptotic effect on regulatory T cells (Tregs), which otherwise suppress effector T cells and a beneficial cellular immune response (see, *e.g.,* enclosed abstract by Simon et al., *Eur. J. Immunol.* 37:758-767, 2007). Therefore, Fas ligand elimination of Tregs also potentiates the above-mentioned anti-tumor immune response observed by the present inventors and described in the specification. Release of beneficial cellular immune responses also provides systemic immunity that protects an individual from metastases, speaking to one of the Examiner's concerns regarding metastatic cancers. The Examiner should also note that these data, which include the data in the present specification, demonstrate that not all tumor cells in a tumor have to be infected by the Fas ligand-encoding vector to be killed as a result of the administration of the Fas ligand, speaking to many of the Examiner's concerns about gene delivery.

Accordingly, the specification demonstrates that expression of Fas ligand by cells at or adjacent to the site of the cancer is effective to reduce tumor burden in a mammal (*i.e.,* reduce tumor size, growth, or metastases or eliminate tumor cells). Taken together, these experiments demonstrate that a recombinant viral vector encoding Fas ligand is effective for reducing or eliminating tumor growth and size of tumor cells by apoptotic mechanisms, some of which in

turn lead to inflammatory mechanisms and protective immunity, thereby also reducing metastases from the cancer cells.

Enclosed herewith are several post-filing publications that confirm the specification results and provide further demonstrations that administration of a nucleic acid molecule, and particularly a viral vector, encoding Fas ligand, or a cell that has been transduced with such a nucleic acid molecule, is effective *in vivo* to reduce tumor burden. These publications show that the present invention has continued to be validated by the inventors' own subsequent experiments, as well as by other investigators.

The publication of Bianco et al. (*Cancer Gene Therapy*, 10:726-736, 2003) shows that Fas ligand gene therapy using naked DNA is safe in tumor-bearing dogs (that approximate human cancer patients), and also that the disease-free interval and survival in dogs that received Fas ligand was equal to or greater than that seen in historical controls treated with standard-of-care. Accordingly, this publication shows that Fas ligand delivered via gene therapy methodology is effective *in vivo* in spontaneously occurring tumors in another animal (malignant melanoma in dogs), and also addresses the Examiner's expressed concern regarding the safety of administering Fas ligand to a mammal.

This publication also references other publications demonstrating that anti-tumor immunity is generated as a result of Fas ligand administration that renders the subjects resistant to subsequent tumor challenge (see Discussion). Again, this latter discussion emphasizes the point that it is not necessary that all tumor cells at the site of a tumor be infected with Fas ligand in order to be killed as a result of the administration of the Fas ligand (*i.e.,* Fas ligand works both by direct tumor killing and indirect killing via the inflammatory response and immune system).

These mechanisms and the use of adenovirus-Fas ligand for cancer therapy are also discussed in the enclosed publication of Modiano et al. (*Gene Therapy and Molecular Biology* 10:31-40, 2006). This publication (see page 32, col. 2) emphasizes that adenovirus-based Fas ligand gene transfer for cancer therapy has been shown to be effective, both via Fas-mediated apoptosis of tumor cells and potentiation of inflammation leading to tumor cell death (both described in the specification). These mechanisms of action notably also minimize persistence of FasL in the system (page 32 states that "adenovirus-mediated expression of FasL is extinguished in <2 weeks because transduced cells are killed as a consequence of the

inflammatory response"), and also induce cross-priming that leads to specific, protective antitumor immune responses (page 32, col. 2). This point again addresses both the Examiner's concerns regarding safety and regarding long-term protection and treatment of metastatic cancers.

The present invention has also been subsequently validated by other investigators. Enclosed are publications by Aoki et al. and Zheng et al., and abstracts by Li et al., ElOjeimy et al., Wang et al., Shinoura et al., and Sudarsan et al., that provide additional evidence that administration of a viral vector encoding Fas ligand is effective or is expected to be effective to induce apoptosis at or adjacent to the site of a cancer, thereby resulting in reduction or elimination of tumor cells (*e.g.,* by reduction of tumor size or tumor cell growth), and accordingly reducing the occurrence of metastases from the cancer.

Aoki et al. (*Molecular Therapy* 1:555-564, 2000) shows that adenoviral Fas ligand reduces leiomyosarcoma growth *in vivo* (See also page 564, col. 2, which states that Fas ligand may be effective enough to eliminate tumors even if gene transfer efficiency is not complete).

Zheng et al. (*World J. Gastroenterol.* 11:3446-3450, 2005) shows that adenovirus Fas ligand gene transfer inhibits human gastrocarcinoma tumor growth *in vivo* in nude mice (notably, this experiment primarily measure direct tumor killing effects due to the use of nude mice).

ElOjeimy et al. (abstract, *Cancer Gene Therapy* 13:739-745, 2006) demonstrates that adenovirus Fas ligand suppressed tumor growth *in vitro* and *in vivo* in a model of head and neck cancer.

Li et al. (abstract, *Clin. Cancer Res* 13:5463-5473, 2007) demonstrates that adenovirus Fas ligand has anti-tumor efficacy *in vivo* against prostate cancer and bone tumors.

Shinoura et al. (abstract, *Curr. Gene Ther.* 3:147-153, 2003) reviews adenoviral delivery of several apoptosis-related genes, including Fas ligand, for killing glioma cells, and concludes that it is a useful therapeutic approach for the treatment of brain tumors.

Sudarshan et al. (abstract, *Cancer Gene Ther.* 12:12-18, 2005) describes the *in vitro* efficacy of adenoviral Fas ligand for the treatment of bladder cancer, showing that the treatment is cytotoxic to bladder cancer cells that would otherwise be considered Fas resistant, concluding that it is promising for use *in vivo*.

Finally, Wang et al. (abstract, *Hepat. Pancreat. Dis. Int.* 5:505-510, 2006) shows the use

of adenovirus Fas ligand in combination with bcl-2 (an anti-apoptosis gene) to protect a cell expressing Fas ligand *in vivo*, thus further validating the use of cells expressing Fas ligand as vehicles to produce Fas ligand as well as a vehicle for delivery of Fas ligand in a therapeutic method.

With regard to the Examiner's concerns regarding toxicity to the mammal as a result of administration of Fas ligand, it is believed that the above-referenced publications demonstrate that Fas ligand-encoding vectors can be effectively used *in vivo* without substantial toxicity and that the Fas ligand-expressing tumor cells will be eliminated from the system in any event. Moreover, potential side effects can also be minimized or ameliorated using guidance provided in the specification. For example, the specification teaches, and the claims are now limited to, introduction of the Fas ligand at or adjacent to the tumor cells, thus minimizing the potential toxic effects on other cells or tissues. As discussed above and shown in the publications, even local administration of Fas ligand to a tumor will have effects on tumor cells not infected by the Fas ligand, as well as induce lasting tumor immunity against subsequent tumor growth and metastases. In addition, the specification teaches that the recombinant viral vector can be under the control of tissue-specific promoters or inducible promoters (see, *e.g.,* page 31, lines 11-14; or page 33, lines 28-29). Indeed, the attached abstract by Li et al. and the attached publication by Aoki et al., by way of example, show that use of a tissue-specific promoter reduces toxicity of Fas ligand when used to treat tumors, in addition to showing that viral vector delivery of Fas ligand has anti-tumor efficacy *in vivo*. Also, one of skill in the art will appreciate that some toxicity is common to many cancer therapy approaches, but that some negative side effects can be tolerated if the result is reduction or eradication of the tumor.

With respect to the concerns raised by the Examiner regarding gene therapy and the stable expression of transgenes, in the present invention, long term gene expression is not required, nor is it desirable, since once the cancer cells have been reduced or eliminated, continued Fas ligand expression is not required. In a therapeutic approach, the gene can be reintroduced as needed, which is preferable over continuous expression (see, *e.g.,* page 13, lines 13-37). As discussed above, in fact, The Examiner raises concerns about the type of delivery vector chosen, but the claims are already limited to viral vector delivery, which has widely accepted to be an efficient and effective way to introduce a gene to a cell (*e.g.,* see "Molecular

Biotechnology," Second Edition, by Glick and Pasternak, ASM Press, Washington D.C., 1998, pp. 555-590; discussed on page 32 of the specification, as well as the various publications herein that demonstrate that viral vector delivery is effective). In particular, many viral vectors, such as adenoviral vectors, do not integrate into a host cell, and so issues related to stable, long term expression of the gene are not relevant. The goal of the present invention is not to cure a genetic disease (see Office Action, page 7). Indeed, the claims do not claim curing any disease, but rather a straightforward, observable endpoint (apoptosis of cancer cells, reduction in tumor growth or size, or reduction in metastases of the cancer) that will provide a benefit to a patient suffering from cancer, regardless of whether the cancer is eliminated or not.

In summary, the specification, as supported by additional evidence subsequent to the invention, has shown that administration of a viral vector encoding Fas ligand at or adjacent to the site of a cancer can induce apoptosis of the cancer cells, can reduce tumor size, can reduce tumor growth, and even eliminate tumor cells, as well as reduce metastases of cells from the tumor. In view of the foregoing discussion, the Examiner is respectfully requested to withdraw the rejection of Claims 45-50, 54-55[sic] and 64-65[sic] under 35 U.S.C. § 112, first paragraph.

Applicants have attempted to address all of the rejections raised by the Examiner in the April 18 Office Action, and submit that the claims are in a condition for allowance. The Examiner is encouraged to contact the below-named agent at (303) 863-9700 with any further questions or concerns, to expedite the prosecution of this application.

Respectfully submitted,

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Eur J Immunol. 2007 Mar;37(3):758-67. Links

Regulatory T cells inhibit Fas ligand-induced innate and adaptive tumour immunity.

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CD4+CD25+ regulatory T cells (Treg) are known to influence T cell responses to tumours. Here we have explored the role of Treg in inhibiting not only adaptive, but also innate immune responses to tumours. To this end we used a Fas ligand (FasL)-expressing melanoma cell line in a mouse model. In this system, innate immunity is sufficient to reject the tumour. All mice depleted of Treg and challenged with FasL-expressing melanoma remained tumour-free. Investigation of the underlying cellular effector mechanisms revealed that depletion of Treg enhanced an NK cell response capable of tumour lysis. Furthermore, this initial innate immune response primed mice to make an effective adaptive immune response leading to complete rejection of challenge with the parental melanoma. Both antigen-specific antibody and CD4+ T cells were implicated in protection via adaptive immunity. We conclude that removal of Treg and vaccination with whole tumour cells expressing FasL activates multiple arms of the immune system, leading to efficient tumour rejection. These findings highlight a novel role for FasL in inducing innate immune responses that are normally inhibited by Treg and uncover an adjuvant effect of FasL that can be used to stimulate tumour immunity after depletion of Treg.

Cancer Gene Ther. 2003 Apr;10(4):330-9.

Quantification and characterization of the bystander effect in prostate cancer cells following adenovirus-mediated FasL expression.

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Inducing Fas-mediated apoptosis in prostate cancer (PCa) is a promising new therapeutic approach with the potential to overcome delivery issues currently problematic in cancer gene therapy. We have previously demonstrated that a Fas Ligand (FasL) expressing adenovirus (AdGFPFasL(TET)) was able to induce Fas-mediated apoptosis in a panel of PCa cell lines regardless of their Fas-sensitivity as determined by the agonistic Fas antibody CH-11. We now report that AdGFPFasL(TET)-infected cells produce apoptotic bodies and cellular debris that continues to elicit FasL-mediated bystander killing in uninfected neighboring cells. Using light microscopy, we demonstrate that AdGFPFasL(TET)-infected cells release apoptotic bodies and cellular debris into the local environment and that this material will induce bystander killing in Jurkat, PPC-1, and PC-3 target cells, but not in DU145 and K-562 cells. The bystander killing mechanism is mediated through Fas/FasL interaction because it is significantly inhibited if target cells are pretreated with the pan spectrum caspase inhibitor Z-VAD-FMK or the Fas neutralizing antibody ZB-4. Coincubation of PPC-1 target cells with apoptotic bodies and cellular debris (effector material) induce nearly complete target cell killing at a ratio of 1:1 target to effector. Collectively, these data indicate that AdGFPFasL(TET)-infected PCa cells release apoptotic and cellular debris capable of inducing bystander killing in PCa and supports the development of FasL as a gene therapy agent.

PMID: 12679806 [PubMed - indexed for MEDLINE]

Cancer Gene Therapy (2003) 10, 726–736

www.nature.com/cgt

Enhancing antimelanoma immune responses through apoptosis

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We examined the feasibility of using tumor apoptosis at accessible sites to enhance antimelanoma immune responses in a model of spontaneous canine melanoma. We show that priming peripheral blood mononuclear cells with apoptotic melanoma cells significantly enhanced autologous and allogeneic lymphokine-activated killing of tumor cells. Since various pathways required for intrinsic apoptosis are often inactivated in melanoma, we used Fas ligand (FasL) overexpression to promote extrinsic apoptosis. FasL induced apoptosis in five of six cell lines. Each of the susceptible lines, but not the resistant one, expressed Fas mRNA. In addition, direct intratumoral administration of FasL DNA to tumor-bearing dogs was safe, with no adverse events reported over 7 days of observation. A reduction of tumor burden was seen in three of five dogs treated. The reduction of tumor volume was correlated with Fas expression by the tumors, although one dog with a Fas-negative tumor survived for 82 weeks after treatment. Our data show that overexpression of FasL is suitable to promote apoptosis of Fas$^+$ melanomas, and support the notion that priming immune responder cells with apoptotic tumor cells may enhance antitumor responses. The results also suggest that intratumoral administration of FasL offers a safe route for therapeutic gene delivery.
Cancer Gene Therapy (2003) **10**, 726–736. doi:10.1038/sj.cgt.7700625

Keywords: melanoma; apoptosis; Fas; Fas ligand; dogs

Melanomas are tumors that arise from melanocytes or their precursor cells and present as pigmented nodules. The location of these tumors in the dog can be used to predict biological behavior. Tumors on the haired skin are generally benign, whereas tumors in the mouth or nail beds are usually malignant. As is true for its human counterpart, canine malignant melanoma is often incurable due to the inability to manage nonresectable and metastatic forms of this disease.[1] New approaches to treat this cancer are warranted, because the existing therapies for metastatic tumors are inadequate. Furthermore, spontaneous malignant melanoma of dogs provides a suitable model system to evaluate novel therapeutic approaches for human patients with malignant melanoma because the progression of the disease is similar in these species.

Gene therapy has shown some success in the treatment of melanoma in mice, humans, and dogs,[2–5] and "priming" through induction of apoptosis also has been shown to enhance immune responses against both viral and tumor antigens.[6–8] Previous data indicate that various pathways that promote intrinsic apoptosis are commonly inactivated in canine melanoma.[9] Consequently, pro-apoptotic genes that can activate the cell death program irrespective of the genetic make-up of the tumor would be needed to develop a feasible treatment protocol that will re-establish the capacity of tumor cells to undergo apoptosis. In addition, recent data suggest that vigorous inflammatory responses at the site of a primary tumor might promote an antitumor response that can prevent growth of distant metastases.[10,11] Forced overexpression of Fas ligand (FasL) appears to fulfill these criteria.[10–14] Ligation of Fas by FasL, or by anti-Fas antibodies that

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promote Fas receptor multimerization (probably formation of trimeric receptor complexes), results in formation of a death-inducing signaling complex (DISC), which initiates a cascade of events that culminate in activation of caspases 8 and 9 and lead to apoptosis.[15] Some cells are refractory to DISC formation, but in these cells ligation of Fas can also promote apoptosis by amplification of the signal through mitochondrial "activation".[15] The potent proapoptotic properties of FasL make it a desirable agent for cancer therapy. Our group and others have shown that malignant cells that do not express Fas, or that express Fas mutants with signaling defects and are resistant to FasL-dependent apoptosis *in vitro*, fail to form tumors in mice if they are transduced with membrane-bound FasL.[10,11,13,16–21] The unusual antitumor properties of FasL, therefore, appear to offer a major advantage in its application for cancer therapy. For this study, we examined the capability to induce apoptosis of canine melanoma cells *in vitro* by overexpression of FasL, and we assessed the safety of *in vivo* administration of FasL in a Phase I clinical trial that included five dogs with spontaneous malignant melanoma (World Health Organization (WHO) stage III or IV).

Methods

Cells and cell lines

The CML-2, CML-13, JEN, SCO, SHA, BEAR, and TLM1 melanoma cell lines have been described previously.[2,22] Cell lines were also derived from tumor explants from each dog enrolled in the *in vivo* safety trial.[23] The tumors were aseptically minced and mechanically disrupted, digested with trypsin, and separated through a fine mesh to produce a single-cell suspension. These cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Canine peripheral blood mononuclear cells (PBMC) were isolated from anticoagulated whole blood obtained from healthy dogs using discontinuous density gradient centrifugation over Ficoll–Hypaque.[24] Paired cell lines and PBMC that were cryopreserved in liquid nitrogen were available from two melanoma-bearing dogs (JEN, BEAR) that participated in a previous gene therapy trial.[2] To prepare PBMC for cytotoxicity assays, cryopreserved cells were thawed to 37°C, sorted for viability, and cultured overnight as described below. The L1210-Fas cell line is a derivative of the L1210 murine lymphoma cell line engineered to overexpress the Fas receptor. L1210-Fas cells were maintained in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, and 10 mM *N*-2-hydroxyethyl piperazine-*N*-*N*-2-ethane sulfonic acid (HEPES) at a concentration of 250,000 cells/ml.

Chemicals and reagents

Tissue culture materials were obtained from BD-Falcon (Bedford, MA); chemicals were obtained from Sigma (St Louis, MO) unless otherwise specified. Human recombinant interleukin-2 (IL-2) was obtained from Chiron Corp (Emeryville, CA) and Hoffman-La Roche, Inc. through the Biologic Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Cell killing assays

Melanoma cell killing mediated by canine PBMC was performed as described.[24] For allogeneic cytotoxicity assays, PBMC from healthy dogs were cultured (primed) in the presence of five CML-2 cells, serum-deprived apoptotic CML-2 cells,[25] or in the absence of melanoma cells (unprimed) for 20 hours. For autologous cytotoxicity assays, PBMC from each melanoma-bearing dog available for this study (JEN and BEAR) were cultured (primed) in the presence of live cells or serum-deprived apoptotic cells derived from their own tumor, or in the absence of melanoma cells (unprimed) for 20 hours. Primed and unprimed PBMC were then cultured with 5×10^3 ^{51}Cr-labelled target cells in the presence or absence of IL-2 for 18 hours. For the autologous cytotoxicity assays, the recovery of viable PBMC was limiting; thus, killing was examined only at an effector-to-target ratio of 25:1. Assessment of FasL-dependent cytolysis was performed as described.[21] Melanoma cell lines were removed from culture flasks by incubating at 37°C in a trypsin-free chelating solution (135 mM NaCl, 5 mM KCl, 20 mM HEPES, and 1.5 mM EDTA, pH 7.4). These cells were cultured with ^{51}Cr-labelled L1210 cells for 16–20 hours. Percent-specific lysis was calculated using the following formula: $(e-s/m-s) \times 100$, where e, s, and m equal the amount of radioactivity released from melanoma cells incubated with effector cells (experimental lysis), with 100 μl medium instead of effector cells (spontaneous lysis) or with 100 μl of 1% Triton X-100 (maximum lysis), respectively. For assays where multiple effector-to-target ratios were used, data were normalized to lytic units. One lytic unit is the number of effector cells required to kill 1×10^3 target cells.

FasL gene delivery

Murine FasL was delivered into canine melanoma cells by adenovirus-mediated transduction (Ad-mFasL).[21] Cell monolayers were trypsinized and washed once with standard growth medium. Cells were resuspended in 500 μl of medium containing the indicated ratio of replication-deficient viral particles (plaque-forming units or PFU) of Ad-mFasL per cell. Cells were incubated for 1 hour in a 37°C water bath with periodic mixing and were washed twice with medium prior to replating at a density of 1×10^5/ml. Human FasL was transfected into the cells using cationic liposomes (Dosper, Roche Diagnostics, Chicago, IL).[26] A measure of 3 μg of the green-fluorescent protein (GFP) expression vector encoding hFasL was mixed with 9 μg of Dosper in 100 μl of HEPES-buffered saline and incubated for 15 minutes at 25°C. The empty GFP vector was used as a negative control. These mixtures were added in 1 ml of OptiMEM serum-free medium (Gibco BRL) to melanoma cells (3×10^5) that

had been adhered to six-well plates by an overnight incubation. The cell–DNA–liposome mixtures were incubated for 6 hours at 37°C, followed by the addition of 1 ml of complete media and incubation overnight at 37°C. FasL expression was verified by the ability of transduced or transfected cells to kill L1210-Fas mouse lymphoma cells.

Gene expression

Expression of Fas mRNA was determined by RT-PCR. Total RNA was isolated from melanoma cells and PBMC using the RNAwiz kit (Ambion, Austin, TX) as per the manufacturer's instructions. Total RNA (1 μg) was converted to first-strand cDNA using the First Strand Synthesis kit from Roche. For assessment of Fas expression, primers were designed based on conserved sequences of Fas in humans and mice: 5′-catgccatggGGACCCAGAATACCAAGTGCAGATG-3′ (sense) and 5′-ggactagtGGTGTTGCTGGTGAGTGTGCATTC-3′ (antisense), where lower case nucleotides represent restriction sites for cloning. Primers were mixed at a concentration of 5 pM/μl with 10 μl cDNA in 10 × PCR buffer (PCR Core Kit, Roche). Amplification was performed using an annealing temperature of 53°C for 30 cycles. The identification of the amplification product as a partial canine homologue was verified by sequencing. The Genbank accession number is AF536812.

Clinical trial design

The main goal of this trial was to evaluate safety of intralesional administration of the FasL gene, with limited estimates of efficacy as a secondary objective. In order to accomplish this with the smallest number of animals possible, we used a sequential testing strategy aimed at considering early termination in the event that adequate safety was not observed. New participants could only be enrolled in the trial after the results of the previous animal with regard to toxicity (see below) were evaluated. Table 1 outlines the decision-making process for enrollment of each new subject.

Based on the poor outcome for dogs with this disease and the frequency of toxic side effects associated with radiation therapy (part of the standard of care for this disease), we designed the trial to define the toxicity associated with intralesional FasL gene therapy, using the probability that adverse events would occur less than 30% of the time as a benchmark. We calculated the relationship (cumulative probability) of efficacy and the predicted

Table 1 Decision-making process for clinical trial enrollment

Conditional outcome	Decision rule	Rationale
Two dogs show toxic side effects	Consider stopping	The probability of experiencing two dogs positive for toxicity in a row is 0.04 if the toxicity rate is 0.20 and 0.09 if the rate is 0.30 If no other adverse effects are seen and other dogs have been negative prior to two positive dogs, consider continuing; otherwise stop
One of three dogs shows toxic side effects	Consider stopping (after one more dog)	The probability of one in six dogs is 0.393 when the toxicity rate is 0.200 and 0.302 when the rate is 0.30. The probability of one in three when only three dogs are exposed is 0.384 and 0.441 for toxicity rates of 0.20 and 0.30, respectively. Thus, unless other data suggest that the treatment has failed, attempt one more dog. If the result in the third dog is a toxicity, Rule 1 applies, otherwise continue.
If one of four dogs shows toxic side effects	Continue	
If two of five dogs show toxic side effects	Continue	As Rule 1 eliminates the possibility of certain sequences, the only ways two of five dogs can occur (each with a probability of 0.0205 or 0.0309 assuming five trials and toxicity rates of 0.20 and 0.30) are: negative–positive–negative–positive–negative positive–negative–positive–negative–negative positive–negative–negative–positive–negative positive–negative–negative–negative–positive These four combinations would occur over 10% of the time in five dogs with either toxicity rate, so that stopping is considered only if other signs or symptoms suggest failure
If three dogs show toxic side effects	Stop	Only one sequence is possible: positive–negative–positive–negative–positive and this would indicate that the toxicity rate is likely higher than 0.30

number of "safe" events (for 0, 1, 2, ... successes). Success was defined as tumor regression or stable disease (size of tumor less than or equal to the initial size with none or only "acceptable" adverse side effects). Although resulting in a large confidence interval, we determined that a sample size of five to six dogs could provide confidence that the success rate was at least 33%, and possibly as high as 50%, as well as reasonable assurance that the toxicity rate was less than 30%, and provide impetus for additional experimentation.

Toxicity and efficacy measures

Unacceptable occurrence of severe local toxicity or systemic toxicity was evaluated for each dog based on the presence of discomfort (at or near the injection site), pain, necrosis, severe systemic inflammation, acute hepatitis or liver necrosis, renal failure, or myocardial toxicity. Mild inflammatory changes in the complete blood count (CBC) due to the administration of a foreign gene, and "stress leukograms" associated with hormonal influence incited by the hospital environment and the presence of disease (advanced melanoma) were expected and were not considered to be significant in the absence of additional evidence of systemic toxicity. These mild inflammatory changes included neutrophilia ($\leqslant$25,000/μl), lymphocytosis ($\leqslant$10,000/μl), and/or monocytosis ($\leqslant$2500/μl). Stress leukograms would be characterized by neutrophilia ($\leqslant$30,000/μl) with lymphopenia ($\leqslant$1000/μl). Any change in the CBC beyond these, or in combination with biochemical changes described below that could not be attributed to disease progression, would be considered significant. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP), along with the levels of total bilirubin, albumin, and cholesterol were used to assess liver function. The following criteria were used as indicators of hepatotoxicity: two-fold elevations of ALT and AST (concurrently), two-fold elevation in ALT concurrent with four-fold elevation in ALP, four-fold elevation in bilirubin concurrent with two-fold elevation in GGT and >2.5-fold elevation in ALT, or any alteration in enzymes along with decreased albumin and cholesterol. These findings would then trigger an ultrasound-guided liver biopsy, and the histopathological diagnosis would be used to rule out or confirm toxicity, and to enact the stopping rules if applicable. The levels of blood urea nitrogen (BUN), creatinine, phosphorus, and total protein were used to assess renal function. The following criteria were used as indicators of nephrotoxicity: creatinine >1.8 mg/dl, BUN >35 mg/dl, and phosphorus within the reference range. These findings would then trigger evaluation of a new sample within 24 hours. Consistent elevations of BUN and creatinine that could not be attributed to dehydration or disease progression would be considered significant. Hypoproteinemia with severe proteinuria and pyuria was not considered as an indicator of renal damage unless it persisted for more than 2 weeks even with appropriate treatment. Hypoproteinemia with severe proteinuria in the presence of renal casts would be considered as a possible indicator of acute renal damage and re-evaluated within 7 days. Persistent changes that could not be attributed to disease progression would be considered significant. The following criteria were used as indicators of myocardial toxicity: auscultation, electrocardiogram, and the activities of creatine phosphokinase (CPK) and AST. The development of murmurs that were not detected during the original physical exam, conduction abnormalities, or concurrent elevations (>2.5-fold) in CPK and AST (with no changes in ALT) would be considered significant. These findings would trigger radiographic and echocardiographic evaluation of the heart. Evidence of significant change in heart size from baseline, reduced contractility, and other biochemical evidence of heart disease would be considered significant.

These index variables were used to classify a dog as experiencing a toxic result. Thus, if any of the compound parameters for systemic toxicity (liver, kidney, heart, or unacceptable systemic inflammation that could not be attributed to disease progression) showed a significant elevation at either the 3- or 7-day sampling or if an animal died from toxicity attributable to treatment (not due to disease progression), then that dog will be classified as having a toxic reaction. All the data and interpretation by the project investigators and by contract pathologists were subject to review by a Safety Monitoring Committee that included a licensed veterinarian who were not associated with the study. The Safety Monitoring Committee had the power to recommend that stopping rules be activated as needed.

Efficacy was measured based on local effects, including necrosis, inflammation, tumor regression, stable disease, or tumor progression, as well as systemic effects including the disease-free interval and duration of remission.

FasL administration to tumor– bearing dogs

The main criterion for patient inclusion consisted of the presence of measurable oral melanoma (WHO stage I–IV). Five dogs with a provisional diagnosis of oral malignant melanoma (based on clinical signs and cytopathology) were entered into the trial. The characteristics of the research subjects are shown in Table 2. The dog owners were offered participation in the trial as one of the treatment options available that might cause palliation of their dog's disease. All the procedures associated with this trial were approved by the Institutional Animal Care and Use Committees of AMC Cancer Research Center and Colorado State University, and by the Institutional Review Board (for use of human subjects in research as it pertained to dog owners) of AMC Cancer Research Center. The tumors were staged by imaging studies, histological examination, and evaluation for distant metastasis to regional lymph nodes and lungs (Table 2). Immunostaining was performed by IHC Services (Smithville, TX). Complete blood counts and serum biochemistry profiles were carried out to assess anesthetic risk and to rule out the presence of additional

Table 2 Signalment of dogs enrolled in Phase I clinical trial[a]

	Breed	Age (years)	Sex	Tumor location, and WHO stage
Dog 1	Min. Schnauzer	12	FS	Palate, stage III
Dog 2	Mix breed	13	MC	Left mandible, stage III
Dog 3	Mix breed	7	FS	Zygomatic arch extending to occipital bone[b]
Dog 4	Mix breed	11	MC	Palate, stage III
Dog 5	Min Poodle	13	MC	Palate, stage III

[a]Dogs of any age, breed, and sex with oral melanoma (WHO Stage I–IV) were eligible for enrollment. WHO stage III includes patients where the primary tumor is >4 cm in diameter (T3), or patients with tumors of any size where there are moveable ipsilateral lymph nodes that contain tumor (any T, N1b), but where there are no distant metastases (including distant nodes).

[b]A final diagnosis of melanoma was confirmed for dogs 1, 2, 4, and 5. The tumor in dog 3 had morphological features that were consistent with a poorly differentiated, nonpigmented, spindle cell tumor. The differential diagnoses included amelanotic melanoma and osteosarcoma, but the cells did not express markers of melanocytic (Melan A, S100) or osteoblastic (osteocalcin) differentiation, leading to a final diagnosis of poorly differentiated sarcoma.

medical problems. Anesthesia was induced using acepromazine and glycopyrrolate for sedation, followed by catheterization of a cephalic vein and intravenous administration of propofol. Anesthesia was then maintained with inhaled Isoflurane. Routine wedge biopsies of the tumors were obtained. Next, 600 μg of hFasL-GFP dissolved in 1 mg of cationic liposomes (Lipofectin®, Life Technologies, Bethesda, MD through an *in vivo* license held by Roche, Palo Alto, CA) was delivered by injection of five to seven sites in and around the tumor in a volume of approximately 1 ml of sterile balanced salt solution (0.9% NaCl) using 22-g needles. Recovery from anesthesia was monitored by certified veterinary technicians and dogs were discharged to their owners on the same day. Narcotic analgesics were used for pain control as needed. Dogs were required to return 3 days after the initial treatment for a full physical examination, complete blood count, and serum biochemistry panels, and 7 days after the initial treatment for a physical exam, complete blood count, serum biochemistry panels, and initiation of standard of care therapy (surgery and/or radiation).

Results

Priming canine PBMC with apoptotic cells enhances antitumor cytotoxicity

We first examined the capacity to induce priming through apoptosis under allogencic conditions. An effective and reproducible method to induce apoptosis in many cultured cells, including canine melanoma cells, is serum withdrawal for 3–4 days.[25] Thus, CML-2 cells were cultured in complete media or in serum-deprived media for 3 days prior to priming. Then, canine PBMC were cultured without priming, or primed by incubation with viable CML-2 cells or apoptotic CML-2 cells for 20 hours, after which primed and unprimed PBMC were cultured with viable ^{51}Cr-labelled CML-2 cells (targets) to determine the extent of cytotoxicity in the presence or absence of IL-2, adjusting the effector-to-target ratios from 25:1 to 100:1. The results show that, under these conditions, unprimed canine PBMC exhibited detectable natural killer cell activity toward allogeneic melanoma cells (105 ± 20.9 lytic units), and that cytolysis was enhanced in the presence of IL-2 (149 ± 13.9 lytic units), possibly through generation of LAK cells.[24] The ability of PBMC to kill CML-2 targets was reduced when these cells were primed using viable CML-2 cells (64 ± 19.4 lytic units), although the cytolytic activity was restored in the presence of IL-2 (172 ± 24.4 lytic units). More importantly, the ability of PBMC primed using apoptotic cells to kill the CML-2 target cells was unaffected in the absence of activating cytokines (107 ± 16.6 lytic units); yet the activity of these cells in the presence of IL-2 was significantly greater ($P < .05$) than that seen for the other conditions tested (274 ± 34.7 lytic units).

To verify that the priming effects were not simply due to allogeneic reactions (i.e., major histocompatibility complex mismatches between the normal PBMC and the CML-2 cells), we used paired canine PBMC and melanoma cell lines that were available from a previous study.[2] Viable autologous PBMC were cultured with each cell line under the same conditions described above. The recovery of PBMC from each dog was limiting, so assessment of cytotoxicity was limited to an effector-to-target ratio of 25:1. JEN or BEAR cells were cultured in complete media or in serum-deprived media for 3 days prior to priming. Then, autologous PBMC were cultured without priming, or primed by incubation with the corresponding viable cells or apoptotic cells for 20 hours, after which primed and unprimed PBMC were cultured with viable ^{51}Cr-labelled targets to determine the extent of cytotoxicity in the presence or absence of IL-2. Figure 1 shows that PBMC from both dogs had only marginal cytolytic activity against the respective autologous melanoma cells, and IL-2 did not enhance cytolysis in either case, possibly due to poor recovery of natural killers cells after cryopreservation. As was seen under allogeneic conditions, the cytolytic activity of these PBMC was not significantly affected by priming with viable tumor cells. In one of the two dogs (BEAR), priming with viable cells significantly increased cytolysis in the presence of IL-2 ($P < .01$). Priming with apoptotic cells alone also did not promote cytolysis of melanoma cells by autologous

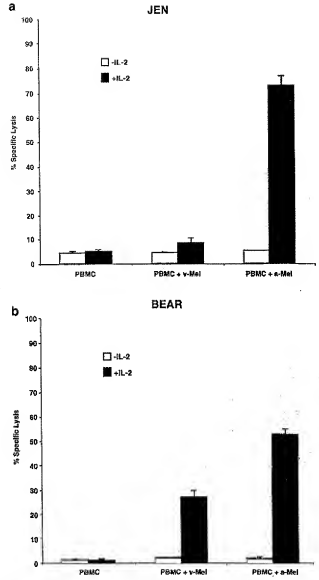

Figure 1 Priming canine PBMC with apoptotic cells enhances antitumor cytotoxicity. PBMC isolated from two dogs with oral melanoma (JEN and BEAR) were incubated alone in complete media, or with viable (v-Mel) or apoptotic (a-Mel) melanoma cells derived from each dog's tumor for 20 hours. The unprimed or primed PBMC (effector cells) were then incubated in the presence of the corresponding autologous melanoma cells (target cells) labelled with ^{51}Cr in the presence or absence of IL-2 for 16 hours. The effector-to-target (E:T) ratio used for these experiments was 25:1; percent-specific cytotoxicity was determined as described in Materials and methods. The cytotoxic activity of PBMC incubated with apoptotic melanoma cells in both dogs, and with viable melanoma cells in the case of BEAR was significantly higher ($P<.005$ and $P<.01$, respectively) than that seen in the control conditions.

PBMC, but PBMC from both dogs primed with apoptotic cells showed significant enhancement of cytolytic activity ($P<.005$) in the presence of IL-2 when compared to every other condition tested.

Induction of apoptosis of canine melanoma cells by FasL

Ligation of Fas with FasL is an effective means to induce apoptosis of many cell types.[27] Based on previous data indicating that various molecules that promote apoptosis using the intrinsic pathways are commonly inactivated in canine melanoma,[9] we examined the capacity to promote apoptosis of these cells by overexpression of FasL as a means to activate the extrinsic pathways.[14] FasL was transduced into six distinct canine melanoma cell lines by infection with replication-deficient adenoviruses encoding murine or human FasL. To ensure that apoptosis was not mediated by the adenovirus vector, cell lines were also transfected with a plasmid encoding human FasL using cationic liposomes,[26] as well as with a control adenovirus vector expressing only the GFP tag. Infection of the cell lines was verified microscopically by assessment of GFP fluorescence. As shown previously,[25,26] melanoma cells transfected with empty vectors showed negligible apoptosis. Each of the transduced (Fig 2a) or transfected (not shown) cell lines expressed detectable levels of FasL as

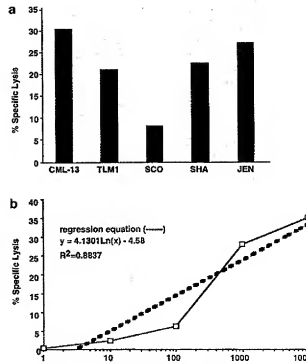

Figure 2 Overexpression of FasL in canine melanoma cells. Adenovirus-mediated transduction of murine FasL was used to overexpress the gene in CML-13, TLM1, Scooter (SCO), Shadow (SHA), and Jenny (JEN) cell lines. A total of 100,000 cells from each line were infected with Ad-m-FasL at an m.o.i. of 1000 viral particles/cell. **(a)** The expression of FasL was examined in each cell line by its ability to kill Fas-bearing L1210-Fas target cells at an E:T ratio of 0.5:1. **(b)** Relationship between FasL expression and m.o.i. in the FasL-resistant JEN cell line.

determined by their ability to kill L1210-Fas target cells. However, only five of the six cell lines underwent apoptosis (affecting nearly 100% of the cells) upon expression of FasL as determined by microscopic examination of cellular morphology, whereas one cell line (JEN) was resistant. Increasing the multiplicity of infection (m.o.i.) in this cell line showed that there was a log-linear relationship between m.o.i. and FasL expression to >1000 viral particles per cell (Fig 2b), but the corresponding increase in FasL expression failed to induce apoptosis of this cell line.

Loss of Fas expression confers resistance to FasL-mediated apoptosis

Resistance to FasL-mediated killing could be due to loss of Fas expression by the tumor cells, or to inhibition of signalling pathways that operate downstream of Fas as is the case in the murine Lewis lung carcinoma cell line[28] (J Sun and D Bellgrau, unpublished). To investigate the first possibility, we cloned a fragment of canine Fas that shares ~78% homology with the corresponding Fas fragments of primate, rodent, and ruminant origin. We then used this gene fragment as a marker to analyze Fas gene expression by RT-PCR. Each of four susceptible melanoma lines analyzed had detectable levels of Fas mRNA, but the resistant cell line did not (Fig 3), indicating that Fas expression was required for FasL-mediated apoptosis.

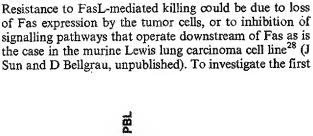

Figure 3 Expression of Fas mRNA by canine melanoma cell lines. Fas expression was examined by RT-PCR as described in Materials and methods. The expression of β-actin was used as a loading control for the reactions and to ensure integrity of the RNA. Normal canine PBMC were harvested without stimulation or were stimulated by PHA followed by restimulation with IL-2 every 3 days for 10 days prior to harvesting. Human Kit-225 human leukemia cells were maintained in culture in the presence of IL-2. The predicted sizes for the Fas and β-actin amplification products were 146 and 318 bp, respectively. The smaller visible bands in the gel represent primer dimers.

Safety and efficacy of FasL administration to tumor-bearing dogs

The data described above suggest that Fas$^+$ melanoma cells are susceptible to FasL-mediated cell killing. Administration of FasL to tumor cells *in vivo* has been shown to mediate both direct (Fas-dependent apoptosis) and indirect (immune-mediated tumor cell killing) tumor cell death.[10,11,14,18–21] We used a 7-day delay of therapy trial to determine if intratumoral administration of FasL was safe in tumor-bearing dogs. Each of five dogs was given 600 μg FasL cDNA mixed with 1 μg cationic liposomes delivered in 1 ml into viable areas of the tumor. Dogs were monitored for 1 hour or until complete recovery and then were re-evaluated at 3 and 7 days after the procedure. There were no adverse events observed over the course of 7 days, and three of five tumors showed measurable regression (Table 3 and Fig 4). Cells isolated from the two tumors showing the most dramatic responses had detectable Fas expression, whereas those isolated from the tumor showing the weakest response and those isolated from a tumor with no measurable response had no detectable Fas mRNA (Table 2 and Fig 5). At that time, each dog was provided standard of care therapy as indicated for each tumor (surgery, radiation, or palliation; Table 4). The median survival for dogs with stage III oral melanoma that undergo standard of care is as short as 16 weeks, and usually less if the tumor is nonresectable; death is usually disease-related.[29–31] In this group, two dogs achieved complete remission (CR), two dogs achieved partial remission (PR), and one dog had no response. The two dogs that achieved CR died of

Table 3 Clinical response of cancer-bearing dogs treated with FasL[a]

	Local toxicity (inflammation at or near injection site)	Systemic toxicity (constitutional signs or changes in hematological parameters)	Measurable response (day 7)	Fas mRNA expression
Dog 1	None	None	Stable disease (no regression or progression)	ND[b]
Dog 2	None	None	58% reduction in tumor volume	Yes
Dog 3	None	None	NA[c]	No
Dog 4	None	None	12.5% reduction in tumor volume	No
Dog 5	None	None	23% reduction in tumor volume	Yes

[a]Toxicity measures were evaluated immediately after administration of the gene therapy (local reactions), and at 3 and 7 days after treatment (systemic changes). Tumor volumes were measured in three dimensions using graded calipers.
[b]ND=not done. Biopsy samples were insufficient for analysis and a cell line could not be established.
[c]NA=not available. An objective response could not be measured for this dog because there was no visible external tumor, and pretreatment computed tomography images were not obtained.

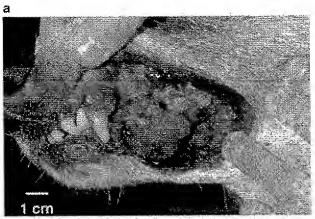

Figure 4 Effect of FasL gene therapy in a dog with oral melanoma. (**a**) Dog 2 at presentation after the tumor was surgically debulked, and a mass measuring 19 mm × 14 mm × 1 mm was left for administration of gene therapy. (**b**) Dog 2 at day 7, when the tumor measured 14 mm × 8 mm × 1 mm. The dog was subsequently treated with surgery (hemi-mandibulectomy) and radiation therapy. Bars = 1 cm.

unrelated causes while free of disease at 24 and 44 weeks post-treatment (Table 4). One of the two dogs that achieved PR died of unrelated causes at 13 weeks with stable disease; the other dog that achieved PR showed evidence of progression at 42 weeks. The tumor was again surgically debulked and the dog that remained maintained good quality of life until it died of progressive disease 82 weeks after the initial treatment. While the expression of Fas by the tumors correlated with the initial magnitude of

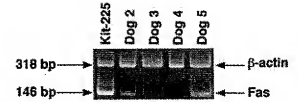

Figure 5 Fas mRNA expression in tumors from dogs treated with FasL gene. Fas expression was examined by RT-PCR as in Figure 2. The expression of β-actin was used as a loading control for the reactions and to ensure integrity of the RNA.

regression, it did not appear to influence response to therapy or survival time. Based on these data, we can say that if the serious adverse event (SAE) rate is truly 30%, the probability that we would observe no SAE is 0.168 and the probability that zero SAE would occur in five dogs if the rate is 40% is 0.078 (7.8% probability that zero of five dogs have a severe reaction).

Discussion

We used malignant melanoma of dogs as a model to evaluate the feasibility of using apoptosis of tumors at accessible sites as a means to enhance antimelanoma immune responses. Malignant melanoma occurs spontaneously in dogs and humans, among other species. Although there are some differences in the initial manifestations of the disease (i.e., the tumor in dogs occurs most commonly in the epithelium of the oral cavity and seems to be unrelated to exposure to ultraviolet light), the progression of disease between humans and dogs is remarkably similar.[1] The disease is highly metastatic in both species, spreading to regional lymph nodes and to the lungs. Also, the response of advanced melanoma WHO stage III or IV) to conventional therapy in these two species is equally dismal. The 5-year survival rate for human patients with disseminated disease is less than 5%;[32] the median survival for dogs with stage III melanoma is 14–16 weeks.[29–31,33] Neither single-agent nor combination chemotherapy is effective to treat malignant melanoma. In dogs, radiation therapy can be palliative and prolong life in cases without metastatic disease.[33] However, in most instances, dogs with oral melanoma (stage I–IV) develop metastatic disease and die within 34–110 weeks of diagnosis. The poor outcomes associated with conventional treatments have prompted investigations of alternative treatments for malignant melanoma, including immunotherapy. Among human cancers, melanoma appears to be uniquely suited to the use of this treatment modality, since this tumor tends to be highly immunogenic.[4,32] Intriguingly, an immunodominant epitope of the Melan A/MART-1 antigen is conserved in dogs,[22,34] and as it is in people, its expression may be prognostically significant.[35]

The use of the biologic response modifiers IL-2 and interferon α (IFN-α) has been evaluated in various human

Table 4 Outcome of cancer-bearing dogs treated with FasL gene therapy[a]

	Treatment	Remission	Survival	Cause of death[b]
Dog 1	Radiation	CR	44 weeks (CR)	Diabetic encephalopathy (no tumor)
Dog 2	Hemimandibulectomy+radiation	CR	24 weeks (CR)	Unknown (no tumor)
Dog 3	Palliative (piroxicam)	None	3 weeks (progressive disease)	Euthanasia (disease-related)
Dog 4	Debulking+radiation	PR	82 weeks (recurrence at 42 weeks; progressive disease)	Euthanasia (disease-related)
Dog 5	Debulking	PR	13 weeks (stable disease)	Euthanasia (after trauma, unrelated to disease)

[a]Follow-up was carried out by the attending veterinarian and by telephone interviews with the owners.
[b]The cause of death for dogs 1, 3, and 5 was verified by a licensed veterinarian. Dog 2 died while sleeping and a necropsy was not performed. The owners reported no visible tumor recurrence in the oral cavity, no palpable lymph nodes present, and no other signs consistent with metastatic disease (e.g., labored breathing, exercise intolerance, inappetence) at the time of death.

clinical trials, with objective responses observed in 20–30% of patients.[4] IFN-α is now an FDA-approved adjuvant therapy for patients with resected stage III melanoma. A preclinical study using human recombinant (hr) IL-2 in combination with hr-TNFα documented tumor regression in five of 13 dogs with malignant melanoma, with acceptable toxicity.[36] Only one of the five dogs, however, had CR lasting more than 3 years.

Myriad additional approaches are under development for human patients with malignant melanoma, including whole tumor vaccines, synthetic vaccines, peptide vaccines, cytokines, dendritic cell vaccines, and DNA or RNA vaccines.[4] Preliminary data from these trials suggest variable levels of success ranging from no benefit over conventional therapy to 5-year survival rates as high as 34%. The assessment of immunotherapeutic approaches for dogs with melanoma has been more limited. Three independent trials have examined the delivery of IL-2 or GM-CSF DNA with histoincompatible cells,[5] in combination with staphylococcal enterotoxin B,[2] or in autologous tumor vaccines.[37,38] The objective response rates for these studies were 50, 33, and 20%, respectively. The first two approaches led to median survival times that exceeded the control group or the anticipated survival based on historical data (39 and 24 weeks, respectively).

These data support the use of immunotherapy in the treatment of melanoma. A particularly appealing aspect of this modality is the possibility to use a patient's own immune cells to destroy metastatic or inaccessible tumors. However, a limitation of "traditional" tumor vaccines and treatments directed against specific tumor antigens is that expression of such antigens can be variable in cancer patients. Yet, it may be possible to circumvent this limitation through a treatment approach that induces immunity against antigens that are expressed in the patient's own tumor. We have pursued development of an immunotherapy approach based on the concept that apoptosis and inflammation can be used to enhance immune recognition of endogenous tumor antigens. In principle, induction of tumor cell apoptosis at an accessible site will enhance the load of intact tumor antigens that are processed and presented by APC to immune effector cells. Moreover, the recruitment of cells that can mount an effective antitumor (type 1) immune response could be further enhanced by promoting inflammation as a "danger signal". This in turn might increase the frequency of tumor-specific immune cells that can be activated by systemic immunotherapy to destroy microscopic foci of tumor cells at both the primary site (that might lead to recurrence) and metastatic sites. The mechanistic basis for the use of apoptosis to "prime" the immune system remains incompletely understood. Although it has been suggested that antigens derived from apoptotic cells can silence the immune system as a response that may have adaptive advantages to help avoid autoimmunity,[39–41] experimental evidence shows that phagocytosis of apoptotic cells by macrophages or dendritic cells can lead to processing and presentation of antigens derived from the apoptotic cells. In two previous studies, it was shown that APC could acquire and present viral antigens from apoptotic cells that led to effective, MHC-restricted CTL responses against viable, virally infected cells,[6,7] and the principle of priming APCs with apoptotic tumor cells to enhance antitumor immunity was demonstrated in a model of abdominal carcinomatosis in rats.[8] Syngeneic macrophages were exposed to apoptotic tumor cells or viable tumor cells *in vitro*, followed by injection (of reisolated, purified activated macrophages) into tumor-bearing rats (with abdominal carcinomatosis). Those macrophages that were exposed to apoptotic tumor cells induced a cure in 80% of the animals when combined with systemic administration of IL-2. In contrast, no animals were cured when given macrophages exposed to viable tumor cells even in the presence of IL-2. Furthermore, the cured animals were resistant to subsequent tumor challenge, and spleen cells from these animals showed remarkable cytolytic activity against tumor cells *in vitro*. The use of FasL as a priming agent offers a unique advantage, since it can not only promote apoptosis of susceptible tumor cells, but also has robust proinflammatory properties that promote rejection of Fas-resistant tumors, as well as protective immune responses to subsequent challenge with those tumors.[10–13,16–18]

For this study, we first examined the effect of priming on canine melanoma cell killing using an established allogeneic system.[24,42,43] The data indicate that the killing activity of PBMC primed with apoptotic cells alone was similar to that of unprimed cells, but, more importantly, priming PBMC with apoptotic cells increased their capacity to kill viable melanoma cells in the presence of IL-2. These results are consistent with recruitment of additional IL-2-responsive cells upon presentation of antigens derived from apoptotic cells. The data also suggest that IL-2 production may have been the limiting factor for the response. Next, we assessed if these effects were also present in autologous conditions, using paired PBMC and melanoma cell lines established from two tumor-bearing dogs. The data show that under autologous conditions, apoptosis induction still offered a remarkable advantage to prime cytolytic activity of PBMC, albeit without abatement of the requirement for IL-2.

Although apoptosis to promote immunologic "priming" can be easily achieved through various means *in vitro*, the development of this principle into a feasible therapeutic approach would require reliable and reproducible induction of tumor cell apoptosis *in situ* using a treatment that has no or limited toxicity. The observation that various pathways required for intrinsic apoptosis are frequently inactivated in canine melanoma[9] led us to consider the possibility of promoting extrinsic apoptosis in these cells using FasL overexpression. The use of FasL was previously shown to be safe by one of our groups in rodents bearing prostatic cancer xenografts;[21] moreover, as noted above, Fas expression by the tumors would not be absolutely required for the treatment to be effective. In our experiments, overexpression of FasL induced apoptosis in each of five Fas-positive canine melanoma cell lines. Predictably, a Fas-negative cell line was resistant to FasL-induced apoptosis *in vitro*. In addition, direct intratumoral administration of FasL DNA to tumor-bearing dogs was safe, with no FasL-dependent adverse events reported over 7 days of observation and a reduction of tumor burden seen in three of five dogs treated. This reduction of tumor volume was correlated with expression of Fas by the tumor cells, although four of the dogs, including at least one that had a Fas-negative tumor, had an objective response (CR or PR) after surgery and/or radiation therapy, and the latter dog survived for 82 weeks after the initial treatment. The dog that did not show a response had a poorly differentiated and advanced tumor (bony lysis was evident from the frontal bone and zygomatic arch to the occipital bone) that was unlikely to respond to any available therapy. While it is possible that the expression of FasL at the tumor site may have sensitized tumor cells in the remaining dogs to the effects of radiation, or promoted nonspecific immune or inflammatory responses that contributed to the observed clinical responses, we could not verify these possibilities based on histological examination of the biopsy sections obtained 7 days after injection of FasL DNA. Nevertheless, the data support the notion that priming immune responder cells with apoptotic tumor cells may enhance antitumor immune responses, and also suggest that overexpression of FasL is suitable to promote apoptosis of Fas$^+$ canine melanomas. Moreover, the results also suggest that intratumoral administration of FasL *in vivo* provides a safe route for therapeutic gene delivery.

Acknowledgments

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Naturally occurring translational models for development of cancer gene therapy[#]

Review Article

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Summary

Most cancer deaths occur from metastatic spread of cancer cells. Immunotherapy and gene therapy are appealing modalities to treat cancer, not only because tumors that are resistant to conventional treatment such as radiation and chemotherapy can be treated using immunologic and genetic approaches, but also because these modalities can reach distant metastases and tumors that are inaccessible for conventional treatment. One gene therapy-based immunologic approach that has shown preclinical promise in laboratory animals is the use of Fas ligand (FasL) gene transfer. FasL promotes tumor cell killing directly and indirectly, and it induces reliable antitumor immune responses that protect animals against subsequent tumor challenge. Yet, despite the unquestioned benefits to study mechanistic questions, factors such as size, pharmacokinetic distribution, and route of administration preclude precise extrapolation of safety data from laboratory mice to humans. We have used spontaneous cancers of dogs as intermediaries for translational studies because the size and physiology of dogs, as well as the natural history of homologous tumors in this species resemble those of humans more closely than rodent models created in the laboratory. Here, we use appendicular osteosarcoma (OS) as an example to document clinical and biological

similarities between the disease in dogs and humans. Specifically, we underscore the unique properties of this model to develop therapy approaches prior to translation into clinical trials of human cancer patients.

I. Introduction

The utility of preclinical animal models for therapeutic development is dependent on how well they approximate the human disease in question. In the realm of cancer, rodent models are especially powerful to define the impact of single gene abnormalities in disease pathogenesis, and strains that are susceptible to chemical carcinogens are well suited to explore the benefit of interventions for cancer prevention. Conversely, the accelerated growth rate exhibited by many transplantable and inducible tumors in laboratory rodents can make evaluation of therapeutic strategies for pre-existing disease problematic.

Cancer in dogs occurs spontaneously. The relative lifetime cancer risk is similar in dogs and in humans, and the shared environment between people and their pet dogs offers opportunities to examine cancer etiology and response to treatment under more realistic conditions. Nevertheless, canine models for therapy development must be chosen with care, not only to properly frame the hypothesis to be tested, but also to reflect the disease under study. Here, we review the strengths of naturally occurring canine osteosarcoma (OS) as a model for preclinical development of Fas ligand (FasL) gene therapy in the adjuvant setting.

II. Fas ligand gene transfer for cancer therapy

We recently reviewed mechanistic basis and preclinical data supporting the use of adenovirus-based Fas ligand (Ad-FasL) gene transfer for cancer therapy (Modiano et al, 2004). The fundamental rationale to develop this approach is based on its potential as an adjuvant treatment: the gene is delivered into the tumor environment, where it primes immune effector cells that mediate systemic antitumor immunity. This then leads to destruction of metastatic cells, increasing the likelihood of durable remissions with reduced morbidity of cancer patients. It is especially important to note that Ad-FasL can promote antitumor immunity by two distinct mechanisms, depending on whether or not tumors express Fas receptors and are susceptible to FasL-mediated apoptosis. Specifically, in the context of cancer gene therapy, ectopic FasL promotes Fas-dependent apoptosis of susceptible tumors. In the tumor environment, scavenging of apoptotic cells by antigen presenting cells can lead to cross priming that enhances cytokine production and killing by tumor-specific T cells (Bianco et al, 2003). On the other hand, when expressed in tumors that are resistant to Fas-dependent apoptosis, the ectopic FasL (and possibly the response to the adenovirus vector) initiates robust inflammatory responses that result in tumor cell death. Unmitigated inflammation is seen with transduction of Ad-FasL, probably because of the high levels of local expression achieved with this method. In these conditions, there is extensive apoptosis of neutrophils and macrophages (Hohlbaum et al, 2001; Shimizu et al, 2001), which in turn perpetuates the inflammatory response by recruiting additional leukocytes. The adenovirus-mediated expression of FasL is extinguished in <2 weeks because transduced cells are killed as a consequence of the inflammatory response (Regardsoe et al, 2004). Therefore, both mechanisms (Fas-mediated apoptosis and inflammation leading to tumor cell death) minimize persistence of FasL in the system, but they also promote specific, protective antitumor immune responses (Modiano et al, 2004). We have thus proposed that FasL gene transfer can be used as a "tumor vaccine" without the need to identify or enrich specific tumor antigens, and have reached a feasibility stage where it is essential to determine the risk-benefit relationships of FasL gene transfer used in an adjuvant therapy setting to treat solid tumors. Among other targets, we propose that adjuvant Ad-FasL therapy has vast potential to improve outcomes of pediatric patients with OS. Dogs with the same disease offer a clinically and biologically relevant model for development.

III. Comparative aspects of human and canine osteosarcoma

OS is an exceptional model for novel therapeutic development, as it meets the following criteria. It is a highly metastatic tumor and patients would benefit from improved treatment options; the disease is relatively common, the tumors can be visualized externally or using imaging, the tumor is responsive to immunotherapy, and both Fas-sensitive and Fas-resistant forms of the tumor occur spontaneously. According to the American Cancer Society, about 2,570 new cases of cancer of the bones and joints will be diagnosed in 2005, and about 1,200 deaths from these cancers are expected (Jemal et al, 2005). OS is the most common among these tumors; it also is the most common type of primary bone cancer in dogs, accounting for up to 85% of skeletal tumors (Dernell et al, 2001) with an annual incidence of 6,000 - >8,000 new cases per year (Withrow et al, 1991; Hansen and Khanna, 2004). Except for the age of clinical onset, the natural history of the disease is similar in people and in dogs (**Table 1**). The standard-of-care for appendicular OS includes amputation or limb-sparing surgery, followed by adjuvant chemotherapy. In children, this treatment produces an overall survival rate of ~80%, but event-free survival (EFS) is lower, with only ~60% reaching five years and barely 50% reaching 10 years (Bielack et al, 2002). Despite these encouraging facts, 20% of children diagnosed with OS will not survive five years, as many as 50% may not see the tenth anniversary of their diagnosis, and most will have significant morbidity associated with the disease. Clearly, there is need for options that will improve the outcomes of patients with this disease. The timeframe bracketed by these hallmarks in children represents ~10% of an average adult lifetime, which provides a reasonable basis on which to compare clinical outcome with dogs that have bone tumors, where the

Table 1. Comparative aspects of human and canine OS[a]
Common recurrent abnormalities are shown in **bold,** those with prognostic or predictive value shown in red.

Clinical Features	**Human**	**Dog**
Age at diagnosis	Adolescent (peak at ~15 yr) (Gurney et al, 1999)	Adult (peak at ~8 yr) (Gorlick et al, 2003; Hansen and Khanna 2004)[b]
Gender-based prevalence	Male ~1.2X	Male ~1.5X
Site	Long bones of limb (78%)	Long bones of limb (85%)
Phenotype	Aggressive, metastatic (lungs most common)	Aggressive, metastatic (lungs most common)
Standard-of-care	Surgery + adjuvant chemotherapy	Surgery + adjuvant chemotherapy
Median event-free survival	~5 yr (<10% of a lifetime)	~9.6 months (<10% of a lifetime)
Pathogenetic Features		
Cytogenetics		
Karyotype	• Aneuploid • Complex to chaotic (Ozisik et al, 1994; Batanian et al, 2002; Bayani et al, 2003; Gorlick et al, 2003; Lopez-Guerrero et al, 2004)	• Aneuploid (Fox et al,; Setoguchi et al,) • Complex to chaotic (Thomas et al, 2005)
Numerical abnormalities	• Gains and losses identified in all autosomes and X chromosome • Chromosome gains outnumber losses by 20-30% • Gain of HSA 19 or loss of HSA 9 predict poor therapy response (Sztan et al, 1997; Friedmann et al, 2002; Gisselsson et al, 2002; Ozaki et al, 2002; Overholtzer et al, 2003; Squire et al, 2003; Lau et al, 2004; Lopez-Guerrero et al, 2004; Man et al, 2004; van Dartel and Hulsebos, 2004; van Dartel et al, 2004; Zielenska et al, 2004)	• Gains and losses identified in many autosomes (Thomas et al, 2005)
Structural abnormalities	• Many chromosomes involved, but disproportionately more frequent with HSA 20 • Many centromeric rearrangements (Ozisik et al, 1994; Miller et al, 1996; Lonardo et al, 1997; Pellin et al, 1997; Kanoe et al, 1998; Yokoyama et al, 1998; Gisselsson et al, 2002; Bayani et al, 2003; Overholtzer et al,; Lau et al,)	• Various rearrangements and centromeric translocations (Thomas et al, 2005)
Oncogenes		
MYC, RAS, HDM2/MDM2, CDK4, MDR-1	• Amplified, mutated, or overexpressed in small number of OS cases • Unknown predictive value or prognostic significance (Nardeux et al, 1987; Ikeda et al, 1989; Barrios et al, 1993; Ladanyi et al, 1993; Antillon-Klussmann et al, 1995; Gamberi et al, 1998; Kanoe et al, 1998; Yokoyama et al, 1998; Ferrari et al, 2004)	• Amplified, mutated, or overexpressed in small number of OS cases • Unknown predictive value or prognostic significance (Kochevar et al, 1990; Mealey et al, 1998; Mendoza et al, 1998)
SIS/PDGFR	• Expression of PDGF AA (*c-sis*) and PDGFR associated with progression and decreased DFI (Sulzbacher et al, 2003)	• PDGF production, PDGFR expression detected in OS cell lines • Low level amplification of *c-sis* in primary OS cases (Kochevar et al, 1990; Levine, 2002)
MET/HGF	• Met overexpression associated with metastatic phenotype • Allelic imbalance at HSA 7q31 is an independent indicator of poor prognosis (Ferracini et al, 1995; Scotlandi et al, 1996; Naka et al, 1997; Oda et al, 2000; Coltella et al, 2003; Entz-Werle et al, 2003)	• Met amplification and HGF co-expression; greater in a pulmonary metastasis (Ferracini et al, 1995; MacEwen et al, 2003) • MET and HGF BACs involved in structural rearrangements
HER2/Neu (ERB-B2)	• *Conflicting data* • Amplification/overexpression detectable in 100% of cases using laser microdissection • Overexpression associated alternatively with higher metastatic potential and decreased DFI, or with increased DFI in different studies (Akatsuka et al, 2002; Anninga et al, 2004; Fellenberg et al, 2004; Ferrari et al, 2004)	• Overexpressed in 4/10 cases of OS and in 6/7 OS cell lines • Overexpression showed trend to decreased overall survival (371 days vs. 487 days) (Flint et al, 2004)
IGF1/IGF1R	• IGF-1/IGF-1R co-expressed in ~50% of primary	• OncoLAR IGF-1 antagonist reduces

	OS • Inhibition of IGF-1R pathway ineffective to slow growth or induce apoptosis, probably due to other autocrine growth loops • OncoLAR IGF-1 antagonist reduces IGF-1 levels but provides no clinical benefit (Burrow et al, 1998; Benini et al, 1999; Mansky et al, 2002)	IGF-1 levels but provides no clinical benefit (Khanna et al, 2002)
CTNNB1 (β-catenin)	• Accumulation of β-catenin in cytoplasm of 33/47 primary OS (Haydon et al,) • ß-catenin-induced activation of LEF-1 inhibits Runx2-mediated osteocalcin expression (Kahler and Westendorf, 2003)	• Unknown, but osteocalcin is frequently undetectable in our samples of primary OS and OS cell lines
Ezrin	• High Ezrin expression associated with metastatic phenotype and poor prognosis (shorter DFI)(Leonard et al, 2003; Khanna et al, 2004)	• High Ezrin expression associated with metastatic phenotype and poor prognosis (shorter DFI) (Khanna et al, 2004)
Tumor suppressor genes		
RB1	• Associated with heritable OS • In sporadic OS, LOH, allelic imbalance, or mutations in 20-70% of cases • Abnormal RB or loss of HSA 13q14 are indicators of poor prognosis (Araki et al, 1991; Scholz et al, 1992; Entz-Werle et al, 2003; Lopez-Guerrero et al, 2004)	• Variable results • Inactivation of Rb, p107, p130 in 1/4 OS lines (Levine and Fleischli, 2000) • Expressed in 21/21 primary OS with no detectable structural abnormalities (Mendoza et al, 1998) • Undetectable in 12/14 OS lines tested by our group
TP53	Associated with heritable risk (Li Fraumeni syndrome) • In sporadic OS, LOH, allelic imbalance, or mutations in 10-80% of cases • Tumors with mutant TP53 have higher level of genomic instability • Abnormal TP53 or loss of HSA17p13 are indicators of poor prognosis (Scholz et al, 1992; Al-Romaih et al, 2003; Entz-Werle et al, 2003; Overholtzer et al, 2003; Squire et al, 2003; Ferrari et al, 2004; Lopez-Guerrero et al, 2004)	• Inactivating mutations in 5/5 OS cell lines and in 8/21 primary OS cases (Mendoza et al, 1998; Levine and Fleischli, 2000)
CDKN2A (p16, INK4A), PTEN	• Inactivated in 30-100% of OS lines or cases • Unknown predictive value or prognostic significance (Nielsen et al, 1998; Ozaki et al, 2002; Park et al, 2002; Entz-Werle et al, 2003; Nielsen-Preiss et al, 2003)	• Inactivated in 5-100% of OS lines or cases • Unknown predictive value or prognostic significance (Levine and Fleischli 2000; Levine et al, 2002; Thomas et al, 2005)
Death receptors		
FAS	• Loss of expression associated with aggressive metastatic phenotype in xenogeneic transplant model (Worth et al, 2002)	• Loss of expression in ~50% of cases with acquired insensitivity to FasL-mediated apoptosis

[a]Only some representative aspects or genes where canine counterparts are known to be affected are shown

[b]In an ongoing study including 65 dogs with primary appendicular osteosarcoma, the peak age at diagnosis was 8-9 years (34/65 cases with known age), the 95% confidence interval of the mean was 7.25 – 8.75 years, and the range was 2-14 years. This is similar to previously reported values (Dernell et al, 2001)

median overall survival in different studies ranged from ~six to ~11 months, with <30% of dogs surviving two years and <10% of dogs surviving three years (Dernell et al, 2001). Since extraneous factors independent of disease can influence overall survival in dogs with OS, a better indicator may be EFS. A recently completed study from one of our research groups (S. Lana et al, unpublished) showed the mean (median) EFS in dogs treated with standard-of-care was 287 (169) days (about 10% of a lifetime).

The efficiency of naturally occurring OS in dogs as a model platform for controlled preclinical study is not only due to higher incidence of the disease in dogs, but also to more rapid progression and apparent similarities in molecular pathogenesis (**Table 1**). Most OS cases in dogs are stage 2b (they present outside the periosteum, have high grade histologic appearance and no detectable metastases). Metastatic disease occurs in >50% of treated animals within one year and in >90% within three years, and it is greater in the lungs than bone. Predictive factors are similar in dogs and people, including age at diagnosis, anatomic location and size of the tumor, histologic grade, serum alkaline phosphatase concentrations, and initial response to therapy (Ehrhart et al, 1998; Dernell et al,

2001; Malawer et al, 2001; Gorlick et al, 2003). Finally, accrual of dogs into clinical studies is rapid, and autopsy compliance is high. For example, a protocol to examine the role of limb-sparing surgery, chemotherapy, and radiation included eligibility criteria of "localized" disease and <50% bone length involvement (Withrow et al, 1993). Forty-nine dogs were accrued, allowing rapid confirmation that, with appropriate candidate selection, this was a suitable treatment option. Interestingly, dogs with infected limb repairs lived twice as long as dogs without infection, suggesting that inflammation at the tumor site with the consequent activation of the immune system has therapeutic benefit. Another randomized study using the insulin growth factor-I (IGF-I) inhibitor, OncoLar, accrued 64 dogs in just eight months (Khanna et al, 2002). In this case, lack of therapeutic benefit of this compound could be confirmed in less than two years. For comparison, 21 people aged between 16 and 35 years old with advanced OS were recruited into a Phase-I multi-institutional study in three years (Mansky et al, 2002). This dose escalation study was stopped due to lack of drug availability when the manufacturer decided toxicities seemed to outweigh clinical benefit. When one considers the accrual rate and the course of disease progression, it might have taken >10 years to show similar negative results in a clinical trial of newly diagnosed (virgin) OS patients.

IV. Molecular features of canine OS and suitability for FasL gene therapy

As noted above, laboratory animal models have a number of limitations that can make translation to humans difficult. Specifically, transplantable tumors or tumors induced by genetic modifications in mice are not always representative of natural tumors. In addition, the homogeneous genetic background in mice strains that can accept tumors (or that develop tumors when exposed to chemicals or when genetic modifications are introduced) do not account for the heterogeneous genetic backgrounds of humans, which can significantly influence tumor progression and response to therapy. Development of better translational models could improve decision-making algorithms to move new therapeutic agents along the development process into clinical trials for human patients. Among all the models available, naturally occurring tumors of dogs present an unparalleled opportunity for use as intermediate steps in the drug development process. Dogs are extensively used in the laboratory setting to define compound safety. However, these controlled conditions still do not approximate the effects that a compound (or a gene) might have in patients that may be debilitated and who may respond differently than healthy individuals. More importantly, cancers of dogs recapitulate the clinical progression of homologous diseases of people, and these animals benefit from participation in clinical studies that can improve their outcome. For studies using canine cancer patients for drug development, safety of the animal "patient" is a major consideration, as the intent is to help these animals in the process of defining a safe (and effective) dose range for the gene therapy that can be translated to humans. Since studies are also designed to identify dose-limiting toxicity or untoward side effects, dog owners recognize there are risks involved (as is true for any clinical study). If toxic events were to occur, this process makes translation more honest and cost-effective because it allows those to be addressed before a Phase-I clinical trial is instituted in human patients (for example, see the case of OncoLar described above).

We have completed preliminary assessments of the suitability to use FasL gene therapy in dogs *in vitro* and *in vivo*. We have established >60 cell lines derived from primary canine OS. These cells grow autonomously in tissue culture, and morphologically they resemble other established canine OS cells lines (Levine and Fleischli, 2000; Liao et al, 2005). The primary tumors and OS cell lines show similar molecular profiles to those seen in humans. For example, they tend to be genetically unstable and have "chaotic" karyotypes (**Figure 1**). We have confirmed the presence of a variety of numerical and structural cytogenetic abnormalities in these tumors, many of which localize to regions that harbor potential oncogenes and tumor suppressor genes (Thomas et al, 2005). In addition, similar gene families seem to be targeted for aberrant expression (activation or silencing) in OS of humans and dogs (**Table 1**).

It is important to remember that a suitable model for FasL gene therapy must provide samples that are susceptible to FasL-mediated apoptosis, as well as samples that are resistant to FasL-mediated apoptosis in order to provide the means to determine if both mechanisms of FasL-induced immune activation provide equivalent clinical benefit, or if case selection would be necessary *a priori* (Bianco et al, 2003). For this reason, we first examined Fas expression in canine OS cells. As was true for melanoma (Bianco et al, 2003), approximately 50% of canine OS expressed Fas (for example see **Figure 2**), and in some tumors, we detected anomalous transcripts. Intriguingly, spontaneous metastases of some of the dogs showed loss of Fas expression, suggesting that, as is true in humans with OS (Worth et al, 2002), loss of this pathway might participate in tumor progression and metastasis.

Previously, we showed that Fas expression by canine melanoma cells correlated with susceptibility to death mediated by transduction with Ad-FasL (Bianco et al, 2003). To verify if this was also true for canine OS, we examined cell viability in culture after transduction with Ad-FasL or Ad-GFP. **Figure 3** shows representative Fas receptor-positive cells (OSCA.36.1) that were susceptible to FasL-mediated cell death, and Fas receptor-negative cells (OSCA2) that were not. The distribution of FasL-sensitive and FasL-resistant OS cells from the lines tested so far is almost exactly 50:50. It is therefore crucial to reiterate the importance of this observation, as it establishes canine OS as a suitable model to confirm the findings in mouse models where tumors that are resistant to FasL-mediated apoptosis *in vitro* are still killed (indirectly) by the inflammation induced by FasL *in vivo*. Also significant is that canine endothelial cells are highly susceptible to adenovirus infection and are killed by Ad-FasL. This minimizes concerns of systemic distribution by

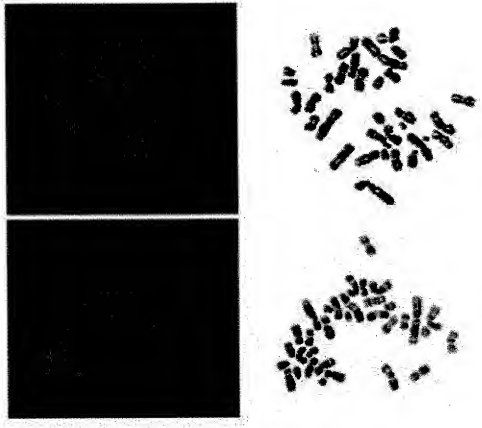

Figure 1. "Chaotic" Karyotypes in Canine OS Cells. The images on the left show DAPI stained metaphase spreads and the images on the right show the corresponding inverted DAPI banded preparations. The modal chromosome number in these cells is significantly reduced (2n=34) compared to normal dog cells (2n=78), and most chromosomes are metacentric, compared to the usual acrocentric morphology of normal canine chromosomes. This 'chaotic' cytogenetic appearance is typical for the canine OS samples we have analyzed (Thomas et al, 2005).

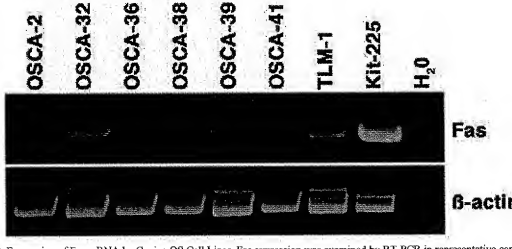

Figure 2. Expression of Fas mRNA by Canine OS Cell Lines. Fas expression was examined by RT-PCR in representative canine OS cell lines established from primary tumor explants. Primers were designed to amplify a 146 bp canine Fas mRNA product. Fas-positive TLM-1 canine melanoma cells and Kit-225 human leukemia cells were used as positive controls; dH_2O without input RNA (in the PCR reaction) was used as a negative control. Expression of ß-actin was used to verify the integrity of the RNA samples and to control for loading differences. (OSCA = osteosarcoma cell line-AMC).

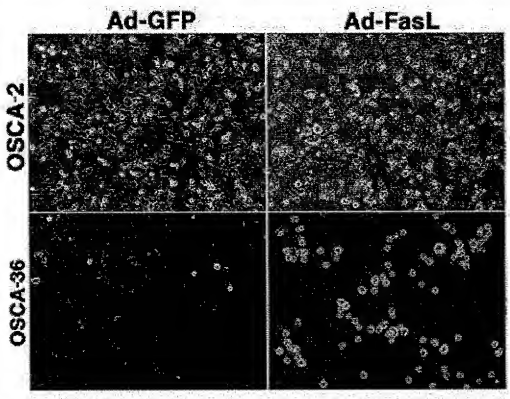

Figure 3. Susceptibility of OS cells to Fas-mediated death. OSCA2 (Fas receptor-negative, top) and OSCA36.1 (Fas receptor-positive, bottom) cell lines are shown to represent, respectively, Fas-resistant cells and Fas-sensitive cells. Subconfluent cultures were transduced using 2,000 pfu of Ad-GFP (left) or Ad-FasL (right). After 6 hr, cultures were photographed under phase-contrast microscopy. While OSCA-2 cells showed virtually no effects of transduction with either adenovirus, more than 90% of OSCA-36 cells showed characteristic apoptotic changes (condensed chromatin, rounded morphology, and detachment from the plastic substrate). OSCA-2 cells continued to grow unhindered in the presence of either adenovirus, as did OSCA-36 cells transduced with Ad-GFP. In contrast, no live cells remained after 24 hr from OSCA-36 cells transduced with Ad-FasL.

the adenovirus after intratumoral administration, as it is likely to remain within the tumor environment where reduced blood flow and increased interstitial pressure at the tumor site (Zachos et al, 2001) will retain the Ad-FasL at or near the injection (within the tumor), promoting transduction of malignant osteoblasts, endothelial cells, and tumor stroma. As also noted previously, we conducted a preliminary study to determine the safety of FasL gene therapy in tumor-bearing dogs using naked DNA (Bianco et al, 2003). No local or systemic toxicity was seen in any of the five dogs in that study. However, based on the preclinical data described above, we are more likely to achieve therapeutic efficacy with Ad-FasL. The safety and toxicity benchmarks for this product will use a more refined method of development that will allow for examination of possible efficacy/toxicity trade-offs if any local (or systemic) toxic events were identified due to the greater levels of FasL expression achieved using the adenovirus delivery system.

V. Conclusions

Naturally occurring tumors of dogs offer unique models that can complement traditional laboratory rodent models for studies of cancer pathogenesis and for preclinical drug development. The strength of these models is the spontaneous occurrence of tumors with similar etiology in large animals that (1) are physiologically similar to humans, (2) share our environment, (3) can tolerate repeated sampling, and (4) largely show comparable responses to conventional treatments. The recent completion of the canine genome sequence (Lindblad-Toh et al, 2005) provides the resources needed to assess the conservation of genes and proteins that can serve as targets for tailored or molecular approaches to treat cancer. We predict that increasingly, studies in pet dogs will become a standard component in the development process of novel therapies for cancer and other chronic diseases, and that these studies will streamline the selection process to determine compounds that have higher a likelihood of success for treatment of human patients. Unquestionably, these studies will also benefit the pet population and provide potential new markets for manufacturers of novel drug and gene-based therapeutics.

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ARTICLE

Restricted Expression of an Adenoviral Vector Encoding Fas Ligand (CD95L) Enhances Safety for Cancer Gene Therapy

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Gene transfer of Fas ligand (CD95L) using adenoviral vectors has been shown to generate apoptotic responses and potent inflammatory reactions that can be used to induce the regression of malignancies *in vivo*, but these vectors also cause significant hepatotoxicity that may limit their clinical utility. Here we describe an adenoviral vector encoding CD95L with restricted gene expression that reduces its toxicity *in vivo*. Preclinical efficacy and gene expression studies of lineage-restricted CD95L adenoviral vectors were performed. To enhance its cytotoxicity and reduce potential systemic effects, a noncleavable CD95L was made by deleting a segment containing the cleavage site (CD95LΔQP). Higher CD95L expression of this mutant was observed on the tumor cell surface, together with a reduction in the release of soluble CD95L. This CD95L cleavage mutant was then expressed under control of a smooth muscle-specific promoter, SM22α, and analyzed for its ability to suppress the growth of tumors of smooth muscle origin *in vivo*. Growth of human leiomyosarcomas but not gliomas was inhibited after ADV gene transfer into tumor-bearing immunodeficient mice. In contrast to viral promoters, in which mortality was uniformly seen after injection of 10^{12} particles, no significant hepatic injury or systemic toxicity was observed in mice, and the maximum tolerated dose was increased ≥10- to 100-fold. These findings suggest that restricted specificity of adenoviral CD95L gene expression enhances the safety of this approach for cancer gene therapy.

Key Words: cancer gene therapy; Fas ligand; CD95L; adenoviral vectors; leiomyosarcoma; SM22α promoter; cleavage mutant; smooth muscle cell.

INTRODUCTION

Fas ligand (CD95L), a member of the tumor necrosis family, is a cytokine that induces apoptosis by binding to its cell receptor, Fas (CD95) (1). CD95L is predominantly expressed in activated T cells and natural killer cells and plays an important role in lymphocyte cytotoxicity (2). The primary function of the CD95–CD95L system is believed to be the immunological self-tolerance and homeostasis of the lymphocyte population (3–6). The dysregulation of CD95–CD95L interaction may contribute to carcinogenesis (7). CD95L is also particularly important in immune-privileged sites such as the eye and testis (8, 9), and CD95L detected on tumor cells may be implicated in immune escape by inducing apoptosis in infiltrating lymphoid cells (10–12). Although it was first believed that myoblasts genetically engineered to express CD95L could serve a general immunoprotective role thereby revolutionizing organ transplantation (13), later studies revealed that ectopic expression of CD95L was instead proinflammatory, yielding potent rejection responses in pancreatic islets rather than protection (14). This result was also observed in muscle; rhabdomyosarcoma cells were rapidly destroyed in the presence of CD95L-expressing myoblasts (15). Thus, the biology of CD95L in immunoprotection and immune tolerance has been demonstrated to be far more complex than previously suspected.

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We have reported previously that CD95L expression on the surface of tumor cells promotes tumor regression through apoptosis or inflammation rather than enhanced tumor growth caused by immune suppression (16). The antitumor effect is dependent on CD95 expression by the tumor cell. In the case of CD95+ tumors, there appeared to be direct induction of apoptosis. In CD95- cells, which are resistant to lysis by CD95L, we and Seino *et al.* observed that tumor regression was induced through an independent mechanism requiring neutrophils that display a "bystander effect" *in vivo* (16–18).

Although *in vivo* gene transfer of CD95L may represent a promising strategy for cancer treatment, the challenge of using CD95L to treat CD95+ or CD95- tumors is complicated by the fact that activation of CD95 induces apoptosis of hepatocytes and liver failure (19–21). Before applying CD95L gene transfer to the treatment of cancers *in vivo*, several issues must be addressed. First, CD95L expression should be restricted to the tumor, because CD95 is expressed in a variety of cells of lymphoid and nonlymphoid origin (22). To achieve this effect in tumors of smooth muscle origin, we assessed whether the SM22α promoter could be used to limit expression of CD95L. This regulatory region, in combination with the 4F2 enhancer, has been shown previously to direct smooth muscle cell lineage-specific expression in adenoviral vectors (23) and could potentially be applied to malignancies of smooth muscle cell origin, such as leiomyosarcomas. Second, the restriction of CD95L expression to tumor cells may not be effective because CD95L expressed on the tumor cell surface is rapidly cleaved, attenuating its cytotoxic activity (24–26). In addition, the function of soluble CD95L is still controversial, with some reports that it may induce toxicity, such as hepatic failure (27). To limit systemic distribution, we constructed a noncleavable CD95L. In this study, we have tested the hypothesis that the combination of a tissue-specific promoter and a noncleavable CD95L would enhance the specificity of adenoviral CD95L expression and reduce toxicity associated with systemic administration.

Materials and Methods

Cells. SK-UT-1B and SK-LMS-1, human leiomyosarcoma cell lines, and CT-26, a mouse colon carcinoma cell line, grown in BALB/c mice, were obtained from American Type Culture Collection. G87, a human glioma cell line, was a gift from P. Kish and K. Murazko (University of Michigan Medical Center, Ann Arbor, MI). M347, a human melanoma cell line, was a gift from A. Chang (University of Michigan Medical Center). The leiomyosarcoma cell lines were grown in MEM supplemented with 10% fetal calf serum, 10 mM nonessential amino acid (GIBCO), and antibiotics. The glioma cell line was grown in DMEM supplemented with 10% fetal calf serum and antibiotics. Other cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. All cell lines are adherent.

Construction of noncleavable CD95L and generation of stably transduced CD95L-expressing cells. A segment coding for 34 amino acid residues at the metalloproteinase cleavage site of human CD95L (residues 103–136) was deleted to create a noncleavable protein designated as CD95LΔQP. CT26 cell lines expressing human CD95L (CT26-CD95L) or noncleavable CD95L (CT26-CD95LΔQP) stably were generated by retrovirus-mediated transduction (28).

Flow cytometric analysis of CD95 and CD95L expression. Target cells (5 × 10⁵) were stained with anti-CD95 antibody (PharMingen) or isotype control IgG followed by fluorescein isothiocyanate (FITC)-conjugated anti-IgG second antibody (PharMingen) or biotin-conjugated anti-CD95L antibody (PharMingen) and streptoavidin–phycoerythrin (PE) to detect the expression of CD95 or CD95L, respectively. Relative fluorescence intensity was measured by fluorescence-activated cell sorter (FACS) analysis of 10⁴ cells.

Adenoviral vectors. The recombinant ADV encoding alkaline phosphatase (AP) and CD95LΔQP was prepared by Cre/loxP-mediated recombination of a sub360 adenoviral cosmid, which is an Ad5 derivative with a deletion in the E3 region, and an AP or CD95L expression plasmid (29). A 441-bp fragment of the SM22α promoter with a *Sal*I site at the 5′ end and *Hin*dIII and *Xho*I sites at the 3′ end was synthesized from murine genomic DNA by PCR. The pAd-SM22α plasmid was generated by subcloning the 441-bp murine SM22α promoter into the *Xho*I site, the 471-bp human 4F2 heavy chain transcriptional enhancer into the *Not*I site, and the 231-bp bovine growth hormone poly(A) signal into the *Xba*I site of the pAdMCSIoxP plasmid (29). pAd-SM22α-AP and pAd-RSV-AP encode the AP gene under control of the SM22α promoter or Rous sarcoma virus long-terminal repeat (RSV-LTR), respectively, and pAd-SM22α-CD95LΔQP and pAd-RSV-CD95LΔQP encode CD95LΔQP cDNA under control of the SM22α promoter or RSV-LTR, respectively. There is a deletion in the E1A and E1B region, impairing the ability of these viruses to replicate and transform nonpermissive cells. Recombinant ADV-mouse CD95L (ADV-CMV-CD95L) and ADV-CMV-AP under control of the cytomegalovirus enhancer/promoter were prepared as described (16).

The propagation of ADV-CMV-CD95L, ADV-SM22α-CD95LΔQP, and ADV-RSV-CD95LΔQP was performed by inclusion of a caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, in the cell culture medium (30). Cesium chloride-purified virus was desalted using sterile BioGel 10DG desalting medium (EconoPac DG10, Bio-Rad) and diluted for storage in a 13% glycerol/PBS solution to yield a final concentration of 1 or 4 × 10¹² particles/ml. All stocks were sterilized by passage through a 0.45-μm filter and evaluated for the presence of replication-competent virus. Using TaqMan PCR analysis (Perkin–Elmer), the titers of replication-competent viruses were less than 3 × 10⁶ particles/ml in 4 × 10¹² particles/ml of recombinant viruses. The ADV deleted of E1 containing RSV-LTR alone (ADV-ΔE1) was used as a negative control.

Annexin V assay. Cultured cells (5 × 10⁵) were prepared by treatment with 2 mM EDTA and then stained with annexin V–FITC (Boehringer Mannheim), which detects phosphatidylserine of an inverted plasma membrane, and examined by FACS analysis.

Gene transfer of CD95L to subcutaneous tumors. Animal experiments were carried out in accordance with both institutional and National Institutes of Health animal care regulations. Seven-week-old female BALB/c nude mice were obtained from Taconic Farms and maintained in a specific pathogen-free environment. As described above, cells were harvested with trypsin, incubated in growth medium at 37°C for 1 h to recover surface molecules, washed, and resuspended in PBS. The cell suspensions (5 × 10⁶ cells per 50 μl) were injected subcutaneously into the flank. The short (*y*) and long (*l*) diameters of the tumors were measured and the tumor volume of each was calculated as $\pi/6 \cdot l \cdot y^2$. For the inoculation of CT26-neo, CT26-CD95L, and CT26-CD95LΔQP cells, groups of six BALB/c mice were used. For ADV-mediated CD95L gene transfer into tumors, 50 μl of viral solution (ADV-RSV-CD95LΔQP and ADV-CMV-CD95L, 1 × 10¹² particles/ml; ADV-SM22α-CD95LΔQP and ADV-ΔE1, 4 × 10¹² particles/ml) was injected into the tumor with a 27-gauge hypodermic needle after the tumor mass was established (~0.4 cm). One mouse from each group was sacrificed for histological analysis of tumor and major organs, and the remaining animals (BALB/c nude, $n = 6$) were observed for tumor growth. For the general distribution of ADV-CD95L, 333 μl of viral solution (3 × 10¹², 3 × 10¹¹, 3 × 10¹⁰, and 3 × 10⁹ particles/ml) was injected intravenously into the mouse (BALB/c, $n = 6$). Intravenous injections were carried out via the tail vein using a 27-gauge needle. Three mice in each group were sacrificed for serum chemical and

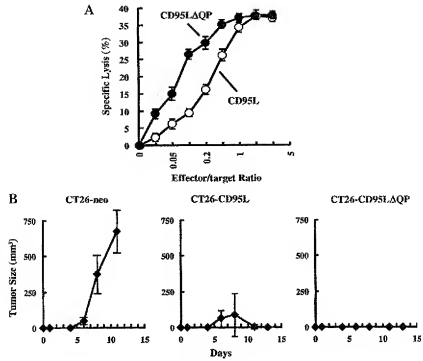


FIG. 1. Noncleavable CD95L has greater cytotoxic activity than wild-type CD95L. (A) Effect of a CD95L cleavage mutant on CD95+ Jurkat cells. The cytotoxic activities of CT26 transformants expressing wild-type CD95L (○) or noncleavable CD95L (●) were determined by using ^{51}Cr-labeled Jurkat cells as a target at the indicated ratios of effector (E/T) cells, as described previously (16). The assays (performed in triplicate) were repeated a minimum of two times and the average numbers plotted with the standard deviation. The ratio of spontaneous ^{51}Cr release to maximum ^{51}Cr was between 12.9 and 16.3%. (B) Regression of CT26 tumors expressing CD95L. The vector-transduced control CT26 cell line (CT26-neo), wild-type human CD95L-expressing CT26 line (CT26-CD95L), and noncleavable human CD95L-expressing CT26 line (CT26-CD95LΔQP) were inoculated subcutaneously into BALB/c mice. The cell suspensions (2×10^6 cells/50 μl) were injected subcutaneously into the flank. The short (l) and long (l) diameters of the tumors were measured and the tumor volume of each was calculated as $\pi/2$.

histological examination 3 days and 1 month after adenoviral injection. The histology of the major organs (brain, lung, heart, liver, spleen, kidney, pancreas, stomach, small intestine, ovary, uterus, urinary bladder, skeletal muscle) was examined by microscopic observation of hematoxylin/eosin-stained slides by an experienced pathologist (L.M.A.).

RESULTS

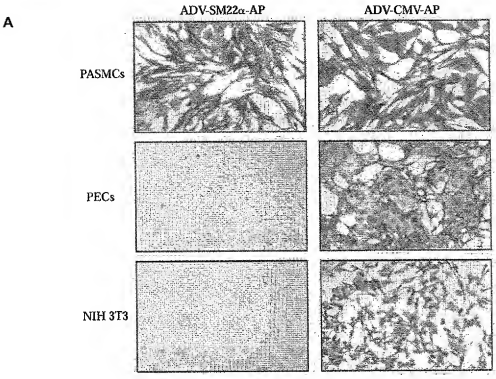
A Mutant Noncleavable CD95L Displays Increased CD95-Mediated Cytotoxicity

An uncharacterized metalloproteinase cleaves the 40-kDa membrane-bound CD95L to generate a 26- to 29-kDa soluble fragment (24–26). We have deleted a 34-amino-acid sequence spanning the metalloproteinase cleavage site of CD95L (residues 103–136), creating a noncleavable protein, CD95LΔQP (28). In agreement with previously published results, processed soluble CD95L was detectable in the supernatant of CT26-CD95L cells, but CT26-CD95LΔQP cells did not produce soluble CD95L, as measured by Western blot analysis of supernatant (24–26, 28). By flow cytometry, CT26-CD95LΔQP showed higher levels of surface expression of CD95L than did CT26-CD95L (28), and CT26-CD95LΔQP showed a high level of cytotoxic activity, comparable to CT26-CD95L, in a ^{51}Cr release assay in Jurkat cells (Fig. 1A).

Tumorigenicity of transfected CT26 cells was evaluated in BALB/c mice. Mice were injected subcutaneously with CT26-CD95LΔQP, CT26-CD95L, or CT26-neo cells. Animals injected with CT26-neo cells rapidly developed large tumors, while those injected with CT26-CD95L developed smaller tumors which spontaneously regressed by day 11. Mice injected with CT26-CD95LΔQP cells did not develop measurable tumors (Fig. 1B).

Transgene Expression from the SM22α Promoter Is Restricted to Smooth Muscle Cells in Vivo

The CD95L cleavage mutant was expressed under the regulation of a smooth muscle cell-specific promoter, SM22α, to restrict the expression of the gene to cells of this lineage. To confirm that the SM22α promoter



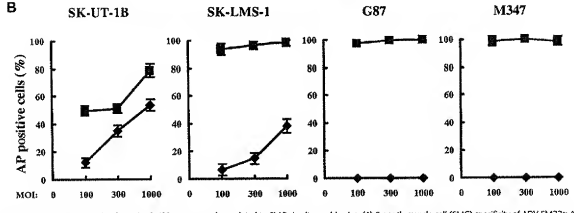
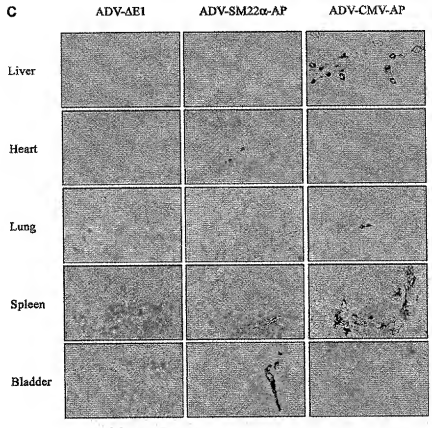


FIG. 2. Transgene expression from the SM22α promoter is restricted to SMCs in vitro and in vivo. (A) Smooth muscle cell (SMC) specificity of ADV-SM22α-AP after *in vitro* gene transduction. Primary porcine aortic smooth muscle cells (PASMCs), primary porcine endothelial cells (PECs), and a murine fibroblast cell line (NIH 3T3) were infected with either ADV-SM22α-AP or ADV-CMV-AP (m.o.i. = 10^3). Cells were fixed and stained for alkaline phosphatase (AP) activity 24 h after infection. (B) Comparison of the activity of ADV-SM22α-AP or ADV-RSV-AP in leiomyosarcoma, glioma, and melanoma cell lines. Cells were infected with 100, 300, or 1000 m.o.i. of either ADV-SM22α-AP (♦) or ADV-RSV-AP (■), and the percentage of cells expressing AP activity was quantified 24 h after infection. The unstained and AP-stained cells from five representative high power fields were counted in each section and the percentage of AP+ cells was calculated. Data were expressed as average percentages of AP-positive cells. (C) Expression of the AP transgene after intravenous administration of ADV-SM22α-AP. C57B/6 male mice received intravenous injections of 2.5×10^{11} particles of ADV-ΔE1, ADV-SM22α-AP, or ADV-CMV-AP. Four days after infection, the mice were sacrificed and their major organs, such as brain, liver, heart, urinary bladder, lung, spleen, kidney, stomach, small intestine, testis, and skeletal muscle, were stained for AP activity. Representative histological sections of liver, heart, urinary bladder, lung, and spleen are shown.



restricted expression of the AP gene to primary porcine aortic smooth muscle cells (SMCs) (PASMCs), a murine fibroblast cell line (NIH 3T3) and primary porcine endothelial cells (PECs) were infected with 1000 m.o.i. of ADV-SM22α-AP or the ADV-CMV-AP virus. Expression of the AP reporter gene under control of the SM22α promoter was observed in primary vascular smooth muscle cells, but no reporter gene activity was detected in fibroblasts or primary endothelial cells (Fig. 2A). To assess the activity of ADV-SM22α-AP in tumor cells, human leiomyosarcoma cell lines (SK-UT-1B, SK-LMS-1), a human glioma cell line, G87, and a human melanoma cell line, M347, were infected with 100, 300, and 1000 m.o.i. of either ADV-SM22α-AP or ADV-RSV-AP. The fraction of cells expressing histochemically identifiable AP activity was quantified (Fig. 2B). SK-UT-1B cells readily expressed the AP transgene after infection with ADV-SM22α-AP, though it was approximately twofold reduced compared to a nonspecific viral enhancer from Rous sarcoma virus, ADV-RSV-AP. Similarly, expression was readily detected at levels comparably below RSV in another smooth muscle tumor line, SK-LMS-1. In contrast, non-smooth muscle tumors, G87 and M347 lines, showed less than 1% of stained cells with ADV-SM22α-AP, whereas more than 97% of the cells expressed AP activity after infection with ADV-RSV-AP, documenting the expected patterns of gene expression in these tumor cell lines. To determine whether transgene expression under control of the SM22α promoter was restricted to SMCs *in vivo*, 2.5×10^{11} viral particles of ADV-SM22α-AP were injected intravenously into the tail vein. ADV-CMV-AP served as a positive control and ADV-ΔE1 as a negative control. Mice were sacrificed 4 days after injection, and the major organs (brain, lung, heart, liver, spleen, kidney, pancreas, stomach, small intestine, ovary, uterus, urinary bladder, skeletal muscle) were evaluated by histochemical staining. Mice that received ADV-CMV-AP showed diffuse AP staining in the liver parenchyma and focal staining in the lung and spleen. Mice that received ADV-SM22α-AP did not show AP activity in major organs except for detectable expression in vascular structures of the heart, spleen, and urinary bladder (Fig. 2C). Mice that received ADV-ΔE1 did not show any AP activity.

Inhibition of Leiomyosarcoma Tumor Growth by ADV-SM22α-CD95LΔQP in Vivo

To examine the ability of ADV-SM22α-CD95LΔQP to inhibit tumor growth *in vivo*, the SK-UT-1B leiomyosarcoma *in vivo* line was compared to the G87 glioma. Both cell lines expressed CD95 on their surface, as determined by FACS analysis (data not shown). Though ADV-RSV-CD95LΔQP infection induced apoptosis in both cell lines, ADV-SM22α-CD95LΔQP caused apoptosis only in SK-UT-1B but not G87 cells (Fig. 3A), confirming the functional specificity of this vector in relevant target cells. The annexin-positive population seen in the cells infected with ADV-ΔE1 may be due to the pretreatment of EDTA or adenoviral toxicity. To characterize the response of SK-UT-1B cells to CD95L expression *in vivo*, tumors were inoculated subcutaneously into the flanks of BALB/c nude mice. After nodules were established (0.4 cm), tumors were injected with ADV-ΔE1, ADV-SM22α-CD95LΔQP, or ADV-RSV-CD95LΔQP. Injection of 1×10^{11} viral particles of ADV-SM22α-AP resulted in transgene expression in 30–40% of SK-UT-1B tumor cells and 90–95% of ADV-RSV-AP cells (data not shown). Infection with ADV-SM22α-CD95LΔQP or ADV-RSV-CD95LΔQP, but not the ADV-ΔE1 vector control, suppressed the growth of SK-UT-1B tumors (Fig. 3B). Histological analysis after injection of ADV-SM22α-CD95LΔQP revealed massive cell death and intense inflammatory infiltrates in tumor tissue (data not shown). Injection of this vector into G87 cells at the same dose had no effect on tumor growth, whereas injection of ADV-RSV-CD95LΔQP markedly suppressed the growth of G87 tumors (Fig. 3C), thus confirming the specificity of the SM22α-CD95L vector *in vivo*.

SM22α-CD95LΔQP Does Not Induce Hepatic Toxicity after Systemic Administration

To characterize the potential toxicity of ADV-SM22α-CD95LΔQP after systemic administration, different doses of ADV-ΔE1, ADV-SM22α-CD95LΔQP, ADV-RSV-CD95LΔQP, or ADV-CMV-CD95L were injected into the tail veins of mice. All seven mice injected with more than 1×10^{11} viral particles of ADV-CMV-CD95L or all five mice injected with 1×10^{10} viral particles of ADV-RSV-CD95LΔQP died within 2 days of fulminant hepatitis, whereas all mice injected with the same amounts of ADV-ΔE1 or ADV-SM22α-CD95LΔQP survived, although they in some cases appeared lethargic. Animals were sacrificed 3 days or 1 month later to evaluate serum enzyme values and histology of major organs. Both groups of mice injected showed a significant increase in sGOT, sGPT, and alkaline phosphatase levels, and in some mice there was a slight increase in creatinine and BUN (Table 1). Histopathological analysis of hematoxylin/eosin-stained sections showed that the intravenous administration of ADV-CD95L resulted in pathological changes in the liver, lung, kidney, and urinary bladder. Injection of 1×10^{11} viral particles of ADV-CMV-CD95L resulted in marked hepatic damage, including fulminant necrosis, ballooning degeneration, cytoplasmic vacuolation and shrinking of nuclei in hepatocytes, focal cell necrosis, infiltration of mononuclear cells, and interlobular bleeding (data not shown). In the lung, proliferation of the epithelial lining, intraalveolar bleeding, and mononuclear inflammatory infiltration were observed, and in the kidney, there was infiltration of mononuclear cells in the cortex, proliferative changes in the glomeruli, and intratubular bleeding in the medulla. Mice injected with ADV-SM22α-CD95LΔQP showed mild changes in the liver (parenchymal degeneration and infiltration of inflammatory cells around the central vein) and lung (mild inflammation and epithelial proliferation) that were related to the dose of injected virus. The degree of inflammation was comparable to that seen with ADV-ΔE1 (Table 2 and data not shown), suggesting that these effects are related to vector protein and/or low-level ADV gene expression and that toxicities from CD95LΔQP are minimized under control of the SM22α promoter. Renal toxicity (inflammation in the cortex and glomerular proliferation) and damage in the urinary bladder (subendothelial inflammation) were slightly more severe with ADV-SM22α-CD95LΔQP than ADV-ΔE1 (Table 2). These histopathological results were consistent with the serum analysis. No macroscopically detectable injuries of brain, heart, spleen, stomach, pancreas, small intestine, ovary, uterus, or skeletal muscle were observed.

Although the *in vivo* AP staining showed focal expression in blood vessels of the heart, spleen, and urinary bladder, histological examination showed no significant abnormalities in vascular structures of these organs after 1 month (Fig. 4). Possibly, the lack of proinflammatory effect in these structures may be related to the specific cytokines expressed in this microenvironment. It has been reported that vascular smooth muscle cells express

FIG. 3. Regression of SK-UT-1B tumors *in vivo* after ADV-SM22α-CD95LΔQP gene transfer. (A) Apoptosis after transfection of ADV-SM22α-CD95LΔQP into SK-UT-1B cells, but not G87 cells *in vitro*. Cells were infected with ADV-ΔE1, ADV-SM22α-CD95LΔQP, or ADV-RSV-CD95LΔQP (m.o.i. = 10³), and apoptotic cells were analyzed by the annexin V assay and compared to controls 30 h after infection. (B) Growth of SK-UT-1B tumors after adenovirus injection. SK-UT-1B cells (5×10^6) were inoculated into BALB/c nude mice. After the tumor was established (0.4 cm), ADV-ΔE1 ($n = 5$), ADV-SM22α-CD95LΔQP ($n = 6$), and ADV-RSV-CD95LΔQP ($n = 6$) were directly injected into established SK-UT-1B tumor nodules on days 4–10. ADVs were injected with 50 μl of a suspension containing 4×10^{12} (ADV-ΔE1 and ADV-SM22α-CD95LΔQP) or 1×10^{13} (ADV-RSV-CD95LΔQP) particles/ml using a 27-gauge hypodermic needle. The tumor volume of each was calculated as described in the legend to Fig. 1B. (C) Growth of G87 tumors after adenovirus injection. G87 cells (5×10^6) were inoculated into BALB/c nude mice and injected with ADV-ΔE1 ($n = 5$), ADV-SM22α-CD95LΔQP ($n = 6$), or ADV-RSV-CD95LΔQP ($n = 6$) as in Fig. 3B.

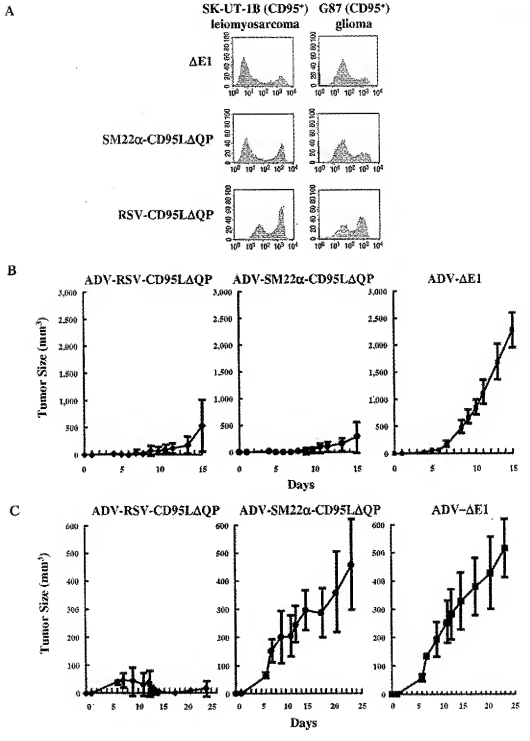


TABLE 1

Evaluation of Selected Serum Enzymes and Chemistries 3 Days (A) and 1 Month (B) after a Systemic Injection of Adenovirus in Mice

A

Vector:	ADV-ΔE1			ADV-SM22α-CD95LΔQP			Control ($n = 6$)
Dose (particles):	10^{10}	10^{11}	10^{12}	10^{10}	10^{11}	10^{12}	-
Alk Phos (IU/liter)	104 ± 20	133 ± 14	214 ± 58 (2)	142 ± 6	105 ± 26	137 ± 11	85 ± 44
Amylase (U/liter)	2284 ± 316 (1)	2288 ± 288 (1)	2297 ± 178 (1)	2309 ± 210 (1)	1842 ± 252	1811 ± 112	1901 ± 201
Bilirubin (mg/dL)	0.3 ± 0.1	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.3 ± 0.1
BUN (mg/dL)	39 ± 4	23 ± 1	20 ± 5	29 ± 5	21 ± 7	15 ± 1	30 ± 7
Creatinine (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0	0.4 ± 0.1	0.3 ± 0	0.5 ± 0.1	0.4 ± 0.2
sGOT (IU/liter)	58 ± 12	70 ± 7	304 ± 204 (2)	55 ± 5	59 ± 12	189 ± 84 (1)	105 ± 54
sGPT (IU/liter)	27 ± 5	28 ± 4	146 ± 105 (2)	23 ± 3	19 ± 1	107 ± 53 (3)	30 ± 5

Vector:	ADV-RSV-CD95LΔQP			ADV-CMV-CD95L		
Dose (particles):	10^{10}	10^{11}	10^{12}	10^{10}	10^{11}	10^{12}
Alk Phos (IU/liter)	130 ± 9	139 ± 31 (1)	†	73 ± 13	†	†
Amylase (U/liter)	2272 ± 85 (1)	2433 ± 526 (1)	†	1743 ± 415	†	†
Bilirubin (mg/dL)	0.3 ± 0	0.3 ± 0	†	0.2 ± 0.1	†	†
BUN (mg/dL)	38 ± 2	23 ± 4	†	27 ± 6	†	†
Creatinine (mg/dL)	0.3 ± 0	0.3 ± 0	†	0.3 ± 0	†	†
sGOT (IU/liter)	110 ± 44	87 ± 21	†	158 ± 3	†	†
sGPT (IU/liter)	35 ± 10 (1)	29 ± 7	†	28 ± 6	†	†

B

Vector:	ADV-ΔE1			ADV-SM22α-CD95LΔQP		
Dose (particles):	10^{10}	10^{11}	10^{12}	10^{10}	10^{11}	10^{12}
Alk Phos (IU/liter)	88 ± 1	76 ± 13	426 ± 194 (2)	108 ± 4	98 ± 21	254 ± 76 (2)
Amylase (U/liter)	1982 ± 196	2065 ± 326 (1)	2432 ± 178 (2)	2319 ± 143	2682 ± 195 (3)	2762 ± 670 (2)
Bilirubin (mg/dL)	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.5 ± 0.3 (1)	0.3 ± 0	0.4 ± 0.1
BUN (mg/dL)	33 ± 7	34 ± 4	31 ± 6	76 ± 71 (1)	34 ± 5	61 ± 34 (2)
Creatinine (mg/dL)	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0	1.2 ± 0.8 (1)	0.7 ± 0.1 (1)	0.8 ± 0.5 (1)
sGOT (IU/liter)	85 ± 16	95 ± 9	517 ± 263 (3)	80 ± 6	92 ± 22	202 ± 34 (2)
sGPT (IU/liter)	32 ± 5	44 ± 6	568 ± 105 (3)	25 ± 7	47 ± 16 (1)	166 ± 27 (3)

Vector:	ADV-RSV-CD95LΔQP			ADV-CMV-CD95L		
Dose (particles):	10^{10}	10^{11}	10^{12}	10^{10}	10^{11}	10^{12}
Alk Phos (IU/liter)	115 ± 5	147 ± 28	†	468 ± 473 (1)	†	†
Amylase (U/liter)	2334 ± 130 (1)	3288 ± 725 (2)	†	2483 ± 311 (2)	†	†
Bilirubin (mg/dL)	0.3 ± 0	0.4 ± 0.1	†	0.3 ± 0	†	†
BUN (mg/dL)	37± 4	34 ± 5	†	28± 4	†	†
Creatinine (mg/dL)	0.6 ± 0	0.6 ± 0.3	†	0.3 ± 0.2	†	†
sGOT (IU/liter)	93 ± 66	143 ± 42	†	110 ± 26	†	†
sGPT (IU/liter)	36 ± 7	96 ± 57 (3)	†	47 ± 12 (2)	†	†

Note. Serum enzyme and chemistry values were analyzed (Antech Diagnostics), and mean values and standard deviations are shown for three mice in each group injected with the indicated adenovirus vectors. Parentheses indicate the numbers of mice that showed abnormal values. † Indicates mortality.

transforming growth factor-β (TGF-β), with transcripts detected as soon as 6 h after vascular injury (31, 32). TGF-β inhibits the proapoptotic and inflammatory effects and may account for the lack of pathology in those vascular structures (33, 34).

Discussion

Although *in vivo* gene transfer of CD95L is a potentially promising strategy for the treatment of cancer, its use in clinical settings may be restricted by its systemic toxicity. In this study, we find that the combination of a specific promoter and a noncleavable form of CD95L enhances its safety. Although membrane-bound human CD95L is cleavable, the site and regulation of proteolysis are not completely defined. Tanaka *et al.* reported that human CD95L was cleaved between Lys129 and Gln130 and constructed a noncleavable CD95L by deletion of amino acids 111–133 or 128–131 (25). Schneider *et al.* reported that the cleavage occurred between Ser126 and Leu127, and the double mutation of cleavage site (Ser126Glu, Leu127Glu) resulted in the loss of detectable

TABLE 2
Organ Pathology † Month after Systemic Injection of Recombinant Adenoviruses into BALB/c Mice

	ADV-ΔE1				ADV-SM22α-CD95LΔQP				ADV-RSV-CD95LΔQP				ADV-CMV-CD95L			
Dose (particles)	10^9	10^{10}	10^{11}	10^{12}	10^9	10^{10}	10^{11}	10^{12}	10^9	10^{10}	10^{11}	10^{12}	10^9	10^{10}	10^{11}	10^{12}
Liver																
Parenchymal degeneration	+(1)	+(2)	+(1)	+++(3)	+(1)	++(2)	++(2)	+(1)	+(2)	+++(2)	++(2)	†(3)	+++(1)	++(2)	†(3)	†(3)
Inflammation	-(3)	-(3)	+(1)	+++(3)	-(3)	-(3)	+(1)	+++(3)	+(3)	+(3)	+(1)	†(3)	+(3)	+(3)	†(3)	†(3)
Individual cell necrosis	-(3)	-(3)	-(3)	+(3)	-(3)	-(3)	-(3)	+(2)	-(3)	-(3)	+(1)	†(3)	-(3)	+(1)	†(3)	†(3)
Lung																
Inflammation	+(1)	+(2)	+++(1)	++(3)	++(3)	++(3)	++(3)	+(3)	++(3)	++(3)	++(3)	†(3)	+++(3)	+++(3)	†(3)	†(3)
Parenchymal degeneration	++(2)	+(2)	+++(1)	+(3)	+(3)	++(3)	++(3)	+(3)	++(3)	++(3)	++(3)	†(3)	++(3)	+++(3)	†(3)	†(3)
Kidney																
Inflammation	-(3)	-(3)	+(1)	+(1)	+(2)	++(1)	+(1)	+++(1)	-(3)	-(3)	+++(1)	†(3)	++(2)	+(2)	†(3)	†(3)
Parenchymal degeneration	-(3)	-(3)	+(1)	-(3)	+(1)	++(1)	++(1)	+(1)	-(3)	-(3)	-(3)	†(3)	+(2)	+(1)	†(3)	†(3)
Urinary Bladder																
Subendothelial inflammation	-(3)	-(3)	-(3)	-(3)	++(1)	+(1)	-(3)	+(1)	-(3)	+++(2)	++(1)	†(3)	-(3)	++(2)	†(3)	†(3)

Note. Each group consisted of three animals and the number of mice showing histological organ changes was indicated in each group. All semiquantitative evaluations were scored blindly from "-" to "+++," where "-" indicates no changes and "+" slight, "++" moderate, and "+++" severe changes. † indicates mortality.

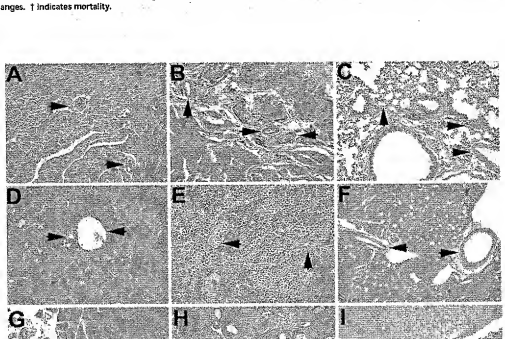


FIG. 4. Analysis of blood vessel walls for organ toxicities. BALB/c mice were systemically infected with ADV-SM22α-CD95LΔQP at a dosage of 1×10^{12} particles. This was the highest dose studied. One month after infection, organ specimens including heart (A), urinary bladder (B), lung (C), liver (D), spleen (E), kidney (F), pancreas (G), ovary (H), brain (I), and stomach and major vessels including aorta, carotid artery, and vena cava were removed, stained with hematoxylin and eosin (H & E), and analyzed for histomorphological changes. Analysis and comparison of small vessel structures in the above-mentioned organs with noninfected or ADV-ΔE1-infected mice showed no vascular abnormalities; there was no medial acellularity and/or necrosis, nuclear changes such as pyknosis and/or condensation, or inflammatory cell infiltration throughout the vessel layers by light microscopy. A slightly increased number of mononuclear inflammatory cell infiltrations was observed in the adventitia of the hepatic arteries and central veins of the portal areas. Thus, adenoviral ADV-SM22α-CD95LΔQP vector administered systemically to mice was not associated with systemic toxicities in the blood vessel walls. Arrows indicate small vessel structures. Original magnification ×200 (A, C, F, and G–I) and ×400 (B, D, and E).

soluble CD95L (26). Based on such studies, we constructed a noncleavable CD95L by deletion of a segment containing amino acids 103–136 (28). This construct could express a high level of CD95L on the surface of cells and induce apoptosis more efficiently than intact CD95L, whose expression level is believed to be rapidly attenuated (25, 26). The noncleavable form of CD95L should therefore enhance cytotoxic activity after *in vivo* transduction at the same time that it reduces systemic release of CD95L. Since the systemic administration of adenovirus expressing CD95L to rats or mice results in widespread hepatocyte apoptosis and death in a dose-dependent manner (19–21), the restriction of CD95L expression in liver is critical for delivery *in vivo*. SM22α is an early marker of SMC lineage that is expressed at high levels exclusively in visceral and vascular SMCs during postnatal development (35–37). Kim *et al.* reported that the arterial SMC-specific SM22α promoter could be used to restrict the activity of ADV to SMCs *in vitro* and *in vivo* (23). Consistent with the previous study, ADV-SM22α-AP was not observed in major organs such as liver and lung, with the exception of focal expression in vascular structures in the heart, spleen, and urinary bladder (Fig. 2*B*). In toxicity studies, mice injected with 1×10^{12} particles of ADV-SM22α-CD95LAQP or ADV-ΔE1 survived, whereas all mice injected with the same dose of ADV-RSV-CD95LAQP and ADV-CMV-CD95L died within 2 days after injection. The results of serum and histological examinations indicate that the ADV-SM22α-CD95LAQP did not enhance hepatic damage when compared to ADV-ΔE1. Recent reports have demonstrated that much of the immunogenicity of adenovirus-infected cells is due to cellular and humoral immune responses directed against the foreign transgene protein (38).

By restricting ectopic expression of adenovirus-encoded transgenes in irrelevant cell types, the ADV-SM22α virus may also reduce immune-mediated damage to organs such as the liver and lung after intentional or inadvertent systemic administration of the vector. It is important to recognize that this approach is also applicable to cancers of other cell types by using different cell-specific regulatory elements in such adenoviral vectors. In this study, SM22α adenoviral vector gene expression using the AP reporter did not reveal activity in the kidney, though renal pathology was observed in some mice injected with high doses of ADV-ΔE1, suggesting possible adenoviral vector toxicity; however, these changes were not associated with long-term renal abnormalities.

Gene transfer of CD95L generated apoptotic responses through the CD95–CD95L pathway and also induced potent proinflammatory reactions through IL-1β release (39) that were used to induce regression of malignancies. It was reported that when parental CD95L$^-$ cells were coimplanted with the same number of CD95L transfectants at the same site, no tumor formation was observed, and even when the ratio of CD95L transfectants to parental cells was decreased to 1/10, tumors grew in only one of three mice, indicating that the *in vivo* bystander effect of CD95L-induced inflammatory effects may be effective enough to eliminate tumors at local sites, even if gene transfer efficiency is not complete (18). Furthermore, CD95L expression after prior immune stimulation in tumors could potentially elicit protective immunity, suggesting that this approach may contribute to a more systemic antitumor response that might eliminate minimal residual disease in clinical settings. Infection with ADV-SM22α-CD95LAQP suppressed the growth of leiomyosarcoma-CD95$^+$ tumor in contrast to the ADV-ΔE1 vector control, and, histologically, infiltration of many inflammatory cells was observed in the tumors. In this study, both mechanisms, proapoptotic and proinflammatory effects, may act to repress tumor growth. Possibly, this approach could be more effective in the treatment of cancer if combined with prior treatment by any number of immune-stimulatory genes or proteins (e.g., allogeneic major histocompatibility complex proteins, superantigens, cytokines, or lymphokines) that might facilitate recognition and elimination of minimal residual disease by the immune system that may not otherwise be eliminated by CD95L gene transfer. TGF-β has been detected in many tumors, and it may inhibit immunologic recognition and rejection of malignancy (33, 40). To enhance the antitumor effect of CD95L gene transfer *in vivo*, it may be required to reverse *in situ* immune suppression by TGF-β.

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• *BRIEF REPORTS* •

Adenovirus-mediated FasL gene transfer into human gastric carcinoma

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Abstract

AIM: To evaluate the possible value of FasL in gastric cancer gene therapy by investigating the effects of FasL expression on human gastric cancer cell line.

METHODS: An adenoviral vector encoding the full-length human FasL cDNA was constructed and used to infect a human gastric cancer (SGC-7901) cell line. FasL expression was confirmed by X-gal staining, flow cytometric analysis and RT-PCR. The effect of FasL on cell proliferation was determined by clonogenic assay, cytotoxicity was detected by MTT assay, and cell viability was measured by trypan blue exclusion. The therapeutic efficiency of Ad-FasL *in vivo* was investigated with a xenograft tumor model in nude mice.

RESULTS: SGC-7901 cells infected with Ad-FasL showed increased expression of FasL, resulting in significantly decreased cell growth and colony-forming activity when compared with control adenovirus-infected cells. The cytotoxicity of anti-Fas antibody (CH-11) in gastric cancer cells was stronger than that of ActD (91±8 *vs* 60±5, $P<0.01$), and the cytotoxicity of Ad-FasL was stronger than that of CH-11 (60±5 *vs* 50±2, $P<0.05$). In addition, G_1-phase arrest (67.75±0.39 *vs* 58.03±2.16, $P<0.05$) and apoptosis were observed in Ad-FasL-infected SGC-7901 cells, and the growth of SGC-7901 xenografts in nude mice was retarded after intra-tumoral injection with Ad-FasL (54% *vs* 0%, $P<0.0001$).

CONCLUSION: Infection of human gastric carcinoma cells with Ad-FasL induces apoptosis, indicating that this target gene might be of potential value in gene therapy for gastric cancer.

Key words: FasL gene; Gene transfer; Apoptosis; Carcinoma; Gastrocellular; Gene therapy

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INTRODUCTION

Gastric cancer is one of the most common digestive tract cancers in China. Although an increasing number of gastric cancer patients have benefited from the development of modern tumor therapies, the prognosis of this disease is still relatively poor. Gastric cancer often resists various treatments, including immunotherapy, wherein deficient tumor-specific T-cell responses result in poor immune response.

In this context, apoptosis mediated by the Fas/FasL system is of great interest to researchers, as cytotoxic T-lymphocytes (CTLs) utilize the perforin/granzyme and Fas/FasL systems to kill cancer cells. Fas is a member of the tumor necrosis factor/nerve growth factor receptor family, whereas the Fas ligand (FasL) is a member of the tumor necrosis factor/nerve growth factor family[1-7]. The FasL is primarily expressed in active CTLs, and induces apoptosis of Fas-expressing tumor cells[1,6]. Since the Fas/FasL apoptosis pathway is a key mechanism for clearing tumor cells, researchers are currently seeking methods for triggering FasL expression in tumor cells via gene therapy, thus 'marking' them for Fas/FasL-mediated apoptosis[5,8]. Accordingly, we used adenoviral gene transfer to trigger high-level FasL expression in SGC-7901 (human gastric cancer) cells to investigate the possible use of FasL in gastric cancer gene therapy.

MATERIALS AND METHODS

Cell line

The human gastric cancer cell line SGC-7901 was obtained from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Medium 199 (Gibco) supplemented with 10% fetal bovine serum.

Recombinant retroviral vector construction

The recombinant FasL retroviral vector was constructed in our laboratory. The FasL gene expression cassette includes the CMV promoter, a full-length FasL cDNA (Jingmei

Company, Shenzheng, China) and the SV40 polyA signal sequences. This cassette was inserted into the E1 region of an adenoviral genome lacking the viral E1 and E3 sequences. Briefly, the FasL cDNA was inserted into the pAdCMV shuttle plasmid (kindly provided by Dr. Daru Lu) and co-transfected with pJM17 (Microbix Biosystems Inc., Canada) into human embryonic 293 cells (provided by Dr. Lu) using the Lipofectin reagent (Gibco). The FasL expression cassette was then transferred into the adenovirus genome by homologous recombination. The control virus Ad-LacZ was constructed in the same manner. Virus proliferation, purification and titering were performed as described by He *et al.*[9].

Adenovirus transduction efficiency

Gastric carcinoma cells were infected with Ad-LacZ at various multiplicities of infection (MOIs). After 48 h, cells were fixed in 40 g/L formaldehyde for 6-8 h and then treated with X-gal solution (1 mg/mL X-gal in a solution of 0.1 mol/L PBS, 1.3 mmol/L $MgCl_2$, 3 mmol/L $K_3Fe(CN)_6$) for 2 h or overnight in a 37 ℃ incubator. The percentage of blue cells was then determined.

FasL gene transfection

Cells were passaged at a density of 10^5/well. For viral infection, cells were incubated with virus suspensions (at various MOIs) and 8 μg/mL polybrene (Sigma) for 2-3 h at 37 ℃, washed twice with fresh medium, and further incubated for 48 h. Then cells were passaged and incubated in medium containing 1 mg/mL G418 (Gibco) for selection of transfectants. The medium was changed every 3-4 d until anti-G418 cells appeared.

Detection of exogenous FasL expression

Cells were harvested, washed with PBS, and treated with FITC-conjugated FasL antibody (Jackson ImmunoResearch Lab) or control serum for 0.5-1 h at 4 ℃. Cells were once again washed with PBS, and FasL protein expression was confirmed by FCM analysis (Becton Dickinson, San Jose, CA). For FasL gene expression, RT-PCR was performed with the kit according to the manufacturer's instructions (Shanghai Shanggong Company, Shanghai, China). The primers were: forward 5'-CTGAATTCTGACTCACCA-GCTGCCATGC-3', reverse 5'-TACTCGAGCTATTAGA-GCTTATATAAGCCG-3'.

Clonogenic assay

SGC-7901 cells were infected with Ad-FasL or Ad-LacZ (100 MOI). After 24 h, the cells were seeded in 6-well plates at 500 cells/well. After being incubated for 2-3 wk, cells were stained with 0.1% crystal violet and counted under a microscope. Colonies of more than 50 cells were counted for all clonogenic assays.

Cell cycle and apoptosis assays

Cells infected with Ad-FasL or Ad-LacZ (more than 10^6 cells) in either suspension or adhesion were harvested and fixed in 70% ethanol for 3 h. Cells were then treated with 50 μg/mL RNase for 1 h at 37 ℃, and stained with 100 μg/mL PI for 20-30 min prior to FCM analysis.

MTT assay

SGC-7901 cells were treated with 100 ng/mL anti-Fas antibody (CH-11; PharMingen), 50 ng/mL actinomycin D (ActD; Sigma), and infected with Ad-FasL (100 MOI) or treated with anti-Fas antibody combined with Ad-FasL infection. Cytotoxicity was determined by MTT assay. About 10^{3-4} cells/well were plated in 96-well plates and incubated overnight in 100 μL of culture medium. After 2-3 d, 20 μL of MTT solution (5 mg/mL) was added to each culture well. After incubating for 4 h at 37 ℃, the MTT was removed and 200 μL of dimethyl sulfoxide (Sigma) was added and mixtures were shaken, the crystals were fully dissolved (about 10 min). The A value of each well was detected using a microculture plate reader (Huandong Cesium Electron Tube Company) with a test wavelength of 490 nm. Cell survival rate (SR) was expressed as the following equation: SR=(A in experimental group/A in control group)×100%. Results were expressed as mean±SD; the Student's t-test was used for statistical analysis.

Cell proliferation assay

SGC-7901 cells ($2.5\text{-}3.0\times10^3$ cells/well) were plated in 24-well plates (3 wells for each test) and infected with various MOIs of Ad-LacZ or Ad-FasL for 24 h. Cells were harvested every 2 d, and the living cell rate (LR) was measured by trypan blue exclusion assay. LR was expressed as the following equation:

LR=number of living cells/(number of living cells+number of dead cells)×100%. Results were expressed as mean±SD; ANOVA was used for statistical analysis with SPSS 10.0 software. $P<0.05$ was considered statistically significant.

Animal model

BALB/c nude mice (The Shanghai Institute of Cancer Research) were subcutaneously injected with SGC-7901 cells at $1\text{-}5\times10^7$ cells/mouse. When tumors grew to 0.5 cm in diameter, the mice were randomly divided into treated and control groups (n = 5). Tumor volumes were calculated by [(1/2)×(longest diameter)×(shortest diameter)] as described previously. Tumors were measured every 5 d for 6 wk. Growth curves were drawn and the percentage of tumor inhibition was calculated (treated group/control group×100%).

RESULTS

FasL gene transfection

Anti-G418 colonies were obtained after screening for 2 wk. The selected clone containing the FasL gene was named as SGC-7901-FasL, and the control clone containing the blank vector was designated as SGC-7901-vect. There was no morphologic difference between cultures of these two cells.

Determination of FasL protein expression

FCM analysis revealed that SGC-7901-FasL cells expressed FasL on their surface, whereas SGC-7901-vect did not (Figure 1).

Determination of FasL mRNA expression

Total RNA was extracted from the test and control cells,

and FasL-specific primers were used for amplification by RT-PCR. The expected 231-bp fragment was amplified from SGC-7901-FasL cells, but not from SCG-7901-vect cells (Figure 2).

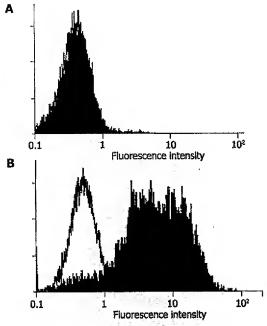

Figure 1 FasL expression on the surface of adenovirus infected SGC-7901 cells. A: Ad-LacZ (titer 4.0×10⁸ CFU/mL); B: Ad-FasL (titer 2.8×10⁸ CFU/mL).

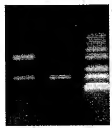

Figure 2 FasL expression as examined by RT-PCR. A: SGC-7901-Fas-L (the FasL amplicon has a size of 231 bp); B: SGC-7901-vect (negative control); C: DNA marker.

Inhibition of SGC-7901 cell growth by exogenous FasL

Two days after infection, SGC-7901-FasL cells were smaller and became more round in shape. Over the next 4 d, the plasma membranes of these cells blebbed, the cytoplasm and nuclei condensed, and the cells ultimately lysed into membrane-bound apoptotic bodies. Cytotoxicity was determined by MTT 4 d after infection. Ad-FasL cultures showed 84% fewer viable cells than Ad-LacZ infected cultures, suggesting that Ad-FasL was cytotoxic to gastric cancer cells. Clonogenic assay showed that SGC-7901 cells infected with Ad-FasL (100 MOI) did not form colonies, whereas Ad-LacZ cultures formed numerous colonies, demonstrating that expression of FasL significantly decreased colony formation.

Cell cycle and apoptosis in Ad-FasL-infected gastric cancer cells

As shown in Table 1, Ad-FasL-infected cultures showed fewer cells in S or G_2M phase, and more cells in G_1 phase, indicating that FasL could inhibit amplification of gastric tumor cells. A sub-G_1 peak (apoptosis peak; Table 1) appeared 4 d after infection of Ad-FasL, indicating that the FasL gene not only induced G_1 phase arrest, but also induced apoptosis of SGC-7901 cells.

Table 1 Influence of FasL expression on cell cycle (FCM analysis) (mean±SD)

Cell lines	G_1/G_0 (%)	S (%)	G_2M (%)
SGC-7901	58.03±2.16	29.72±1.36	9.18±0.92
SGC-7901-vect	61.12±2.24	30.95±1.22	11.02±0.18
SGC-7901-FasL	67.75±0.59[a]	26.56±0.59[a]	5.68±1.12[a]

[a]P<0.05, [b]P<0.05, [c]P<0.05, SGC-7901-FasL *vs* SGC-7901 or SGC-7901-vect.

Cytotoxicity of FasL to SGC-7901 cells

To further evaluate the possibility of gastric cancer gene therapy with Ad-FasL, SGC-7901 cells were infected with Ad-FasL or treated with anti-Fas antibody, and the resulting cytotoxicity was compared with that of ActD, a RNA synthesis inhibitor known as cytotoxin. The cytotoxicity of the anti-Fas antibody (CH-11) to gastric cancer cells was stronger than that of ActD, and the cytotoxicity of Ad-FasL was stronger than that of CH-11 (Table 2).

Table 2 Relative *in vitro* cytotoxicities of various treatments (mean±SD)

Cell line	Treatment group			
	ActD	Ad-LacZ	CH-11	Ad-FasL
SGC-7901	91±8	68±2	60±5[a]	50±2[b]

[a]P<0.05 *vs* CH-11 or (compared with CH-11 or anti-Fas antibody+ActD). [b]P<0.01 *vs* ActD (compared with ActD).

Inhibition of Ad-FasL on tumor growth in animal model

Nude mice were subcutaneously injected with SGC-7901 cells, then intratumoral injections of Ad-FasL were administered. The growth of tumor infected with Ad-FasL was inhibited by 54%, suggesting that Ad-FasL was a viable gene therapy candidate (Figure 3).

DISCUSSION

Although a growing number of tumor patients have benefited from modern oncotherapeutic methods, there is still a need to improve therapies for malignant tumors. Gene therapy is expected to join surgical, radiological and chemotherapeutic strategies in future methods of integrated oncotherapy. Pre-clinical studies have confirmed that adenovirus-mediated high level expression of carcinoma-inhibiting genes (such as p53) can inhibit tumor growth, induce apoptosis and increase

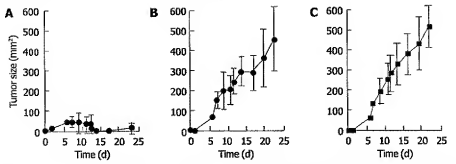

Figure 3 Treatment of SGC-7901 tumor-bearing mice with SGC-7901+FasL cells. **A**: Mice were injected with SGC-7901 cells (5×10^6), followed by treatment with FasL ($n = 8$); **B**: mice were injected with SGC-7901 cells (5×10^6); **C**: mice were injected with SGC-7901 cells (5×10^6), followed by treatment with PBS ($n = 8$). Tumor incidence is presented at each time point in Figure. $P<0.0001$.

tumor tissue sensitivity to radio- and chemotherapy[10-14]. Recent clinical studies in China and abroad have indicated that adenoviral gene therapy is safe and applicable[12-15]. Here, we used this vector to express FasL in cultured human gastric cancer cells. The FasL gene expression cassette includes the CMV promoter, a full-length FasL cDNA, and SV40 polyA signal sequences. A transduction efficiency test using a similarly constructed Ad-LacZ vector illustrated that the adenoviral construct possessed high transduction efficiency. FCM and RT-PCR were used to detect high-level FasL expression in target cells, confirming that the adenovirus vector effectively transfers the FasL gene into tumor cells.

Previous studies[16,17] indicated that binding between FasL and Fas induces receptor trimerization, and apoptosis of Fas-expressing cells. Current theory holds that the signaling responsible for this apoptosis occurs in one of the following three ways: between T cells and target cells, among target cells; or between T cells[18-23]. Fas expression is markedly higher in gastric cancer cells than in normal gastric mucosal cells, implying that Fas participates in the genesis of gastric carcinoma. Fas activation can induce gastric carcinoma cell apoptosis, indicating that the Fas/FasL system might be a good target for gene therapy. In this study, we attempted to induce direct apoptosis of target cells (cis-type apoptosis) by transfecting a highly efficient Ad-FasL expression vector into gastric carcinoma cells (SGC-7901). Expression of FasL inhibited the apoptosis of SGC-7901 cells up to 84%, and significantly inhibited the ability of SGC-7901 cells to form colonies. These results have not been reported in China.

FasL is thought to engage with Fas by inducing receptor trimerization, which then transfers signals to the Fas intracellular death domains (DD). Then, the Fas-associated death domain dimerizes with the DD to transfer an apoptotic signal to Caspase-8, instigating a caspase cascade leading to cell apoptosis[24-26]. In our study, FCM analysis showed that SGC-7901 cells infected with Ad-FasL quickly arrested in the G_1 phase, which was subsequently followed by tumor cell apoptosis. Taken together, these *in vitro* results suggest that FasL gene transfer is capable of inducing gastric tumor cell apoptosis and that it may be a viable candidate for tumor gene therapy.

In our study, an *in vitro* cytotoxicity assay showed that Ad-FasL could significantly inhibit the growth of gastric cancer cells. The inhibition was much stronger than the cytotoxicity conferred by CH-11 treatment, indicating that the Fas/FasL system plays an important role in gastric cancer cell apoptosis[27-29]. In contrast, a SGC-7901 tumor model in nude mice showed only 54% inhibition of tumor growth in response to Ad-FasL infection. This difference between the *in vivo* and *in vitro* response rates might be caused by poor distribution of the recombinant adenovirus in the solid tumor, resulting in lack of target gene transfer to all the tumor cells. Thus, future Fas/FasL gene therapy experiments *in vivo* should focus on stabilizing vectors, increasing transfection efficiency, repeating administration and combining interventional therapy and gene therapy, thereby improving the therapeutic efficacy.

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FasL gene therapy: a new therapeutic modality for head and neck cancer.

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In this study, we investigated the in vitro and in vivo efficacy of Fas ligand (FasL) gene therapy for the treatment of head and neck cancer. Three head and neck squamous cell carcinoma (HNSCC) cell lines (SCC-1, SCC-12, and SCC-14a) were treated with the Fas agonist CH-11, a monoclonal antibody to the Fas receptor, or with a replication-incompetent adenovirus (AdGFPFasL) expressing a modified murine Fas ligand gene fused to green fluorescent protein (GFP). A replication-incompetent adenovirus containing the GFP gene alone was used as a control for viral transduction toxicity (AdGFP). Cell death was quantified using a tetrazolium-based (MTS) assay. Cells were analyzed by flow cytometry to determine the expression of adenoviral and Fas receptors on the surface of the cells. Our results showed that the head and neck cancer cell lines are resistant to cell death induction when treated with the anti-Fas monoclonal antibody CH-11. This resistance can be overcome with AdGFPFasL, which was able to induce cell death in all three cell lines. Apoptosis induction was demonstrated using Western blotting by evaluating poly(ADP-ribose) polymerase, and caspase 9 cleavages. In addition, intratumoral injections of AdGFPFasL into SCC-14a xenografts induced significant growth suppression of tumors, indicating that FasL gene therapy may provide a new efficient therapeutic modality for HNSCC that is worthy of a clinical trial.

PMID: 16543918 [PubMed - indexed for MEDLINE]

Related Links

In vitro efficacy of Fas ligand gene therapy for the treatment of bladder cancer.
[Cancer Gene Ther. 2005] PMID:15514684

Intracellular Fas ligand expression causes Fas-mediated apoptosis in human prostate cancer cells resistant to monoclonal antibody-induced apoptosis. [Mol Ther. 2000] PMID:11020350

The role of adenovirus-mediated retinoblastoma 94 in the treatment of head and neck cancer. [Cancer Res. 2002] PMID:12183420

A novel bystander effect involving tumor cell-derived Fas and FasL interactions following Ad.HSV-tk and Ad.mIL-12 gene therapies in experimental prostate cancer. [Gene Ther. 2002] PMID:11948376

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Fas ligand delivery by a prostate-restricted replicative adenovirus enhances safety and antitumor efficacy.

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PURPOSE: Recent studies showed that Fas ligand (FasL) induced apoptosis in tumor cells and suppressed the immune response in several types of tumors. However, the toxicity of FasL limited further administration. This study delivered FasL in prostate cancer cells using an improved prostate-restricted replicative adenovirus (PRRA), thereby improving the antitumor effect while decreasing systemic toxicity. EXPERIMENTAL DESIGN: We designed a FasL-armed PRRA, called AdIU3, by placing adenoviral E1a and E4 genes, FasL cDNA, and E1b gene under the control of two individual PSES enhancers. Tissue-specific viral replication and FasL expression were analyzed, and the tumor killing effect of AdIU3 was investigated both in vitro and in vivo using androgen-independent CWR22rv s.c. models via local administration and bone models via systemic administration. The safety of systemic administration of AdIU3 was evaluated. AdCMVFasL, in which FasL was controlled by a universal cytomegalovirus (CMV) promoter, was used as a control. RESULTS: AdIU3 enhanced FasL expression in prostate-specific antigen (PSA)/prostate-specific membrane antigen (PSMA)-positive cells but not in PSA/PMSA-negative cells. It induced apoptosis and killed PSA/PMSA-positive prostate cancer cells but spared normal human fibroblasts, hepatocytes, and negative cells. The increase in killing activity was confirmed to result in part from a bystander killing effect. Furthermore, AdIU3 was more effective than a plain PRRA in inhibiting the growth of androgen-independent prostate cancer xenografts and bone tumor formation. Importantly, systemic administration of AdIU3 resulted in undetectable toxicity, whereas the same doses of AdCMVFasL killed all mice due to multiviscera failure in 16 h. CONCLUSIONS: AdIU3 decreased the toxicity of FasL by controlling its expression with PSES, with greatly enhanced prostate cancer antitumor efficacy. The results suggested that toxic antitumor factors can be delivered safely by a PRRA.

PMID: 17875776 [PubMed - in process]

Related Links

Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer. [Cancer Res. 2005] PMID:15753394

A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. [Cancer Res. 2001] PMID:11507044

Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. [Prostate. 2003] PMID:12539223

A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. [Mol Ther. 2001] PMID:11708878

Curr Gene Ther. 2003 Apr;3(2):147-53.

Gene therapy using an adenovirus vector for apoptosis-related genes is a highly effective therapeutic modality for killing glioma cells.

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Preclinical studies in animal models and human clinical trials have evaluated the safety and efficacy of adenoviral vectors for cancer gene therapy. These studies have indicated that gene delivery via adenoviral vectors, including p53 gene therapy, represents a promising therapeutic modality for many types of human cancers. This review focuses on novel strategies to induce apoptosis in glioma cells by transduction with adenoviral vectors carrying a variety of apoptosis-related genes, including Fas ligand, Fas, FADD, caspase-8, p53, p33ING1, p73alpha, Bax, Apaf-1, caspase-9, IkappaBdN, caspase-3, Bcl-2, and Bcl-X(L). We conclude that adenoviral vector-mediated delivery of apoptosis-related genes other than p53 is a potentially useful gene therapy approach toward the treatment of human brain tumors.

Cancer Gene Ther. 2005 Jan;12(1):12-8.

In vitro efficacy of Fas ligand gene therapy for the treatment of bladder cancer.

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Previous investigations have revealed that bladder cancer cells are generally resistant to Fas-mediated apoptosis by conventional Fas agonists. However, the ability of these cell lines to undergo Fas-mediated apoptosis may have been underappreciated. As a result, we investigated the in vitro efficacy of Fas ligand gene therapy for bladder cancer. Three human bladder cancer lines (T24, J82, and 5637) were treated with the conventional Fas agonist CH-11, a monoclonal antibody to the Fas receptor. Cells were also treated with a replication-deficient adenovirus containing a modified murine Fas ligand gene fused to green fluorescent protein (GFP), AdGFPFasL. A virus containing the GFP gene alone was used to control for viral toxicity (AdGFP). Cell death was quantified using a tetrazolium-based (MTS) assay. Cells were also evaluated by Western blotting to evaluate poly (ADP-ribose) polymerase, caspase 8, and caspase 9 cleavage and by flow cytometry to determine the presence of coxsackie/adenovirus receptor (CAR). These studies confirmed bladder cancer resistance to cell death by the anti-Fas monoclonal antibody CH-11. This resistance was overcome with AdGFPFasL at a multiplicity of infection (MOI) of 1000 achieving over 80% cell death in all cell lines. Furthermore, greater than 80% cell death was evident in 5637 cells treated with low-dose AdGFPFasL (MOI=10). 5637 cells expressed significantly higher levels of surface CAR than J82 or T24 cells ($P<.05$). AdGFPFasL is cytotoxic to bladder cancer cells that would otherwise be considered Fas resistant, supporting its in vivo potential. Enhanced sensitivity to AdGFPFasL may be in part due to increased cell surface CAR levels.

1: Hepatobiliary Pancreat Dis Int. 2006 Nov;5(4):505-10. Links

Utility of adenovirus-mediated Fas ligand and bcl-2 gene transfer to modulate rat liver allograft survival.

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BACKGROUND: Expression of Fas ligand (FasL) on the graft by gene transduction is expected to introduce apoptosis to lymphocytes to protect rejection, but the FasL-expressing graft cells may also induce apoptosis as the graft usually expresses Fas antigens. In this study, a strong antiapoptotic gene, bcl-2, was cotransfected with the FasL gene in rat liver graft to protect against Fas-mediated cell death and to prolong recipient survival. METHODS: Orthotopic liver transplantation was done in a strain combination of DA to LEW rats. After donor vascular isolation, adenovirus-mediated FasL and bcl-2 genes were cotransfected in the liver graft. RESULTS: Intragraft expression of FasL mRNA was constitutively expressed after adenovirus-mediated transduction, although expression of FasL increased mildly in control grafts. Bcl-2 mRNA was highly expressed at 2 days after reperfusion. In contrast, lower expression of bcl-2 was observed in the control group. The average survival of the gene transferred allografts increased from (9.8+1.3) days to (18.5+8.7) days compared with the control group. CONCLUSION: Our results indicate that rat liver allografts can be protected against host immune responses by adenovirus-mediated FasL and bcl-2 transfection, and that bcl-2 expression prevents the graft from Fas-mediated apoptosis.

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